The Role of Maternal Age in the Aetiology of Autism Using Population Based Studies

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Abstract

Autism Spectrum Disorders (ASD) are neurodevelopmental syndromes affecting 1%-2% of all children. The aetiology of ASD is unknown, yet evidence supports a role for both genetic and non-genetic, including environmental, factors in aetiology.

This thesis includes four related studies examining the role of maternal factors, and in particular maternal age, in the aetiology of ASD. Using methods of meta-analysis, national Swedish health registers, as well as a multinational cohort combining national registers from five countries this thesis examined: (a) the hypothesis that advancing maternal age is associated with ASD in the offspring ; (b) the hypothesis that maternal reproductive treatments are associated with ASD risk; and (c) the familial risk for ASD.

The meta-analysis provided support for the hypothesis that advancing maternal age is associated with risk for ASD in the offspring. Risk for ASD was 1.3 fold higher (95%CI:1.2-1.4) for offspring of mothers 35 years old or older compared with mothers 25-29. The multinational cohort provided further support for the hypothesis demonstrating an independent effects of paternal and maternal age on risk for ASD. In addition, in addition to the effect of advancing age this study showed an increased risk with increasing differences in age between the spouses.

Fertility treatments, overall, were not associated with risk for autism [RR=1.1; 95% 0.9-1.4]. However, in treatments for the most severe form of male infertility there was a strong association RR=4.6 (95%CI: 2.1-9.9).

Our family study demonstrated that genetic factors explain half of the liability to ASD (h2=50%, 95% CI:). Factors related to maternal intrauterine environment do not seem to play a substantial role in autism aetiology.

In conclusion, maternal age represents a moderate risk factor for autism. The mechanisms underlying this effect may involve both genomic and social factors, and should be rigorously examined in future studies.

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List of abbreviations

AD Autistic disorder (infantile autism)

ASD Autistic Spectrum Disorder

CI Confidence interval

CDC The US Centers for Disease Control and Prevention

DEN Denmark

DSM American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders

ICD International Classification of Diseases

ICARE International Collaboration for Autism Registry Epidemiology

ICSI Intra Cytoplasmic Sperm Injection (sperm is injected directly into an egg)

ID Intellectual Disability (Mental Retardation)

IQ Intelligence quotient

ISR Israel

IVF In-Vitro Fertilization

FIN Finland

GEE Generalized Estimating Equations

MBR Medical Birth Register

MR Mental Retardation (Intellectual Disability)

NOR Norway

PDD Pervasive Developmental Disorders

RR Relative risk (or relative recurrence risk)

SET Single embryo transfer - in IVF when only one fertilized embryo is inserted

SWE Sweden

UK United Kingdom

WAU Western Australia

1 BACKGROUND

This section will describe the Autism Spectrum Disorders. Section 1.1 will describe the diagnostic system of autism spectrum disorders. Section 1.2 will present an overview of the aetiology of autism spectrum disorders and in more detail, in sub-sections, will present selected risk factors of particular importance for this thesis.

1.1 Autism - Diagnosis and origins

1.1.1 Autism characteristics

Autism spectrum disorders (ASD) are a group of severe disorders of brain development. Autism is the most severe form of these disorders. Autism is characterized by social deficits, verbal and non-verbal communication deficits and restricted interests and repetitive behaviours. Autism is evident early on, already by age 2 o -3, and there is considerable individual variation in expression. In addition to the three core components individuals affected with autism can also suffer from intellectual disability and physical and neurological problems.

1.1.2 Diagnostic criteria

The diagnosis of autism is done based on symptomatology and direct observation. There is currently no accepted biomarker for diagnosing autism. In the current DSM IV-R criteria (American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision) autism is part of a wider group of 5 disorders of neurodevelopmental origin, called pervasive developmental disorders (PDD). These include:

1. Autistic Disorder (AD)
2. PDD not otherwise specified (= Atypical autism)
3. Asperger's disorder
4. Rett's disorder
5. Childhood dis-integrative disorder

For a diagnosis of AD the DSM IV-R specify

1. Impairment from at least 2 of 4 items describing social interaction, and,

2. Impairment from at least 1 of 4 items describing communication, and,
3. Impairment from at least 1 of 4 items describing repetitive and stereotyped patterns of behaviour

With at total of at least 6 items from 1-3 above and, importantly, with onset prior to age 3.

The other four diagnosis in the PDD group are characterized by:

- Atypical autism: presentations that does not meet the criteria for AD because of late age at onset or atypical or sub-threshold symptomatology including the "high-functioning" ASD
- Asperger's disorder: differs from the AD category primarily in the lack of significant delay in language or cognitive development and with a later age of onset.
- Rett's disorder: Almost exclusively effecting girls with profound effect on the development and onset usually at 6-18 month of age. As many as 95% of the cases have mutations in the X-linked MECP2 gene (Buxbaum & Hof 2013, p.421).
- Childhood disintegrative disorder is similar to autism in expression but is recognized by a loss of language, social interplay or physical motor skill from an initially normal state. The onset is usually at the age 2-5 but in rare cases as late as age 10. The disorder is very rare.

The full DSM IV-R criteria is described in **appendix A**.

While ICD-10 criteria for Atypical autism is divided into four sub-categories, overall DSM and ICD diagnostic criteria are very similar (see **appendix A**). A bigger problem than the internal comparisons between ICD and DSM is probably the validity of the concept itself considering the lack of specific biological markers (Volkmar 1996).

The terms "autism", "infantile autism", "childhood autism" and "autistic disorder" are often used interchangeably to refer to the same conditions. The term "Autism Spectrum Disorders" (ASD) is used for the AD, Atypical autism and Asperger's Disorder combined.

1.1.3 History of diagnosis

Autism was first described in 1943 by Leo Kanner (Kanner 1968) that described a group of 11 cases (followed up in a paper 30 years later (Kanner 1971)). Even though the observations made by Kanner are still valid today there have been divergent views on how to consider and classify autism. For a period of time autism was considered a subclass under schizophrenia or childhood psychosis (Hollander et al. 2010; Starling & Dossetor 2009) and some even wanted to consider autism a psychogenic disease (Hollander et al. 2010). A variety of studies over the past forty-five years have paved the way for the current view of autism spectrum disorders.

The first major shift in how autism was viewed resulted from the twin studies of the 1970s when it became clear that there are important genetic influence on autism (Susan Folstein & Rutter 1977; S Folstein & Rutter 1977; Bailey et al. 1995). Second, it was concluded that autism was not a part of schizophrenia. The substantial differences in age of onset between autism and schizophrenia made them incompatible as a common disease (Rutter 1972). Third, the difficulty to draw the exact diagnostic borders between different aspects of the disease made the concept of spectrum almost necessary (Wing & Gould 1979).

In the DSM, autism existed as a separate diagnostic entity from 1980 in DSM III and then as "infantile autism" only with 6 characteristics. The 1987 DSM III-R changed the title to “autistic disorder”. The DSM IV, published in the year 1994, extended this to four subtypes consisting of 16 different characteristics.

In the latest version of DSM, the DSM 5 (American Psychiatric Association et al. 2013), ASD was again modified to a single concept where, Asperger's disorder is not mentioned any longer separately, and Rett's disorder has been excluded from the category. A gradient of severity was added with consequences to the need for support.

The discussion on how to define and classify autism still likely continue; together with schizophrenia? (King & Lord 2011) associated with ADHD? (Gargaro et al. 2011) or together with PDD? (Tateno et al. 2011).

1.1.4 Prevalence

The prevalence of autism has increased dramatically over the past 20 years. Although

changes in underlying genetic or environmental causes have not been ruled out, this can not by itself explain the differences in prevalence between different geographical regions. This suggest that there may be a role for social/cultural factors such as awareness and ascertainment, diagnostic substitution and availability of services in prevalence. For instance, it has been estimated that one quarter of the increase in prevalence in California between 1992 and 2005, is due to changes in diagnostic practice(s), e.g. from earlier diagnosis of Intellectual Disability (ID) (King & Bearman 2009). As the age of parenting has been increasing in the United States and Europe in recent decades (Bray et al. 2006; Martin et al. 2006) an association between maternal age and autism may help explain, at least in part, the increase in prevalence estimates of autism during the past two decades. It should also be noted that, by definition, prevalence is a cumulative measure with differences between populations or samples reflecting the differences in age distribution and as such less suited for measuring causal effects than the incidence and the relative risk. In our **studies I, II, III and IV** the aim has been to study aetiology and autism risk factors - not prevalence or society disease burden.

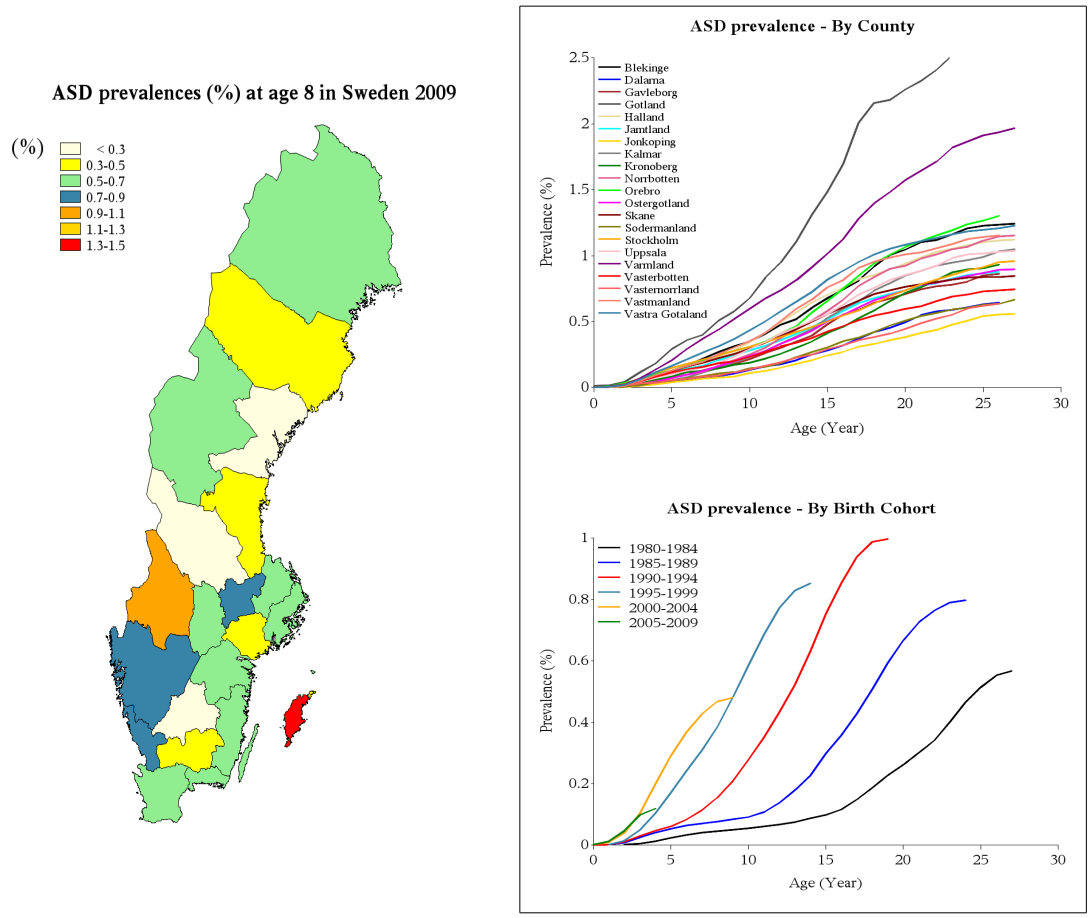
The US Centers for Disease Control and Prevention (CDC) estimate the prevalence of ASD among 8 year old children in the USA to be 1.8% for boys (1 in 54) and 0.4% for girls (1 in 252) (Autism and Developmental Disabilities Monitoring Network Surveillance Year 2008 Principal Investigators & Centers for Disease Control and Prevention 2012).

For the purpose of this thesis, using the data from our **study III** it was possible to estimate the prevalence among all children born in Sweden 1982-2007 and followed up for AD up to 2009. The data show a high heterogeneity between the different counties in Sweden (**Figure 1 Left panel; Figure 1, Bottom right panel**) and with a sharp increase over birth cohorts (**Figure 1, Top right panel**).

The ASD prevalence among 8 year old children in Sweden born 1985-1989 was estimated to 0.08% (95% CI: 0.07-0.09%); born 1990-1994 0.16% (95% CI: 0.15-0.17%) and born 2000-2004 to 0.47% (95% CI: 0.45-0.49%). For AD the corresponding prevalences were 0.06% (95% CI: 0.06-0.07) for children born 1985-89 and 0.28% (95% CI: 0.26-0.29%) for children born 2000-2004.

The ASD prevalence among 8 year old children over all birth cohorts 1980 to 2009 was 0.19% (95% CI: 0.19-0.20%) and for AD 0.12% (95% CI: 0.12-0.13%).

Figure 1 ASD prevalence (%) at age 8 for children born in Sweden 1982-2007. Followed-up for ASD until 31st December 2009



Footnote: Prevalence estimated by inverted Kaplan-Meier curves, 1-S(t), S(t)=Prob(Survive > t| Alive <=t)

1.2 Aetiology

Autism is a genetic disorder, i.e., genetic factors are responsible for a substantial proportion of individual differences in liability to the disorder, and it shares genetic mechanisms with the other PDD (Szatmari et al. 1998). Early twin studies showed a strong heritability of autism with estimate as high as 93% (Freitag 2007; Bailey et al. 1998; Susan Folstein and Rutter 1977; Cichon et al. 2009), and very different

concordance rates for MZ and DZ twins (S Folstein and Rutter 1977; Bailey et al. 1995). For a broader ASD definition the MZ concordance rate in the twin studies was 90% while the concordance rate for DZ twins was below 10% (Bailey et al. 1995).

An epidemiological study of 943,000 children of which 818 developed autism 1994-2001 show a strong association between autism and psychiatric history in mothers (RR=4.0), psychiatric history in fathers (RR=1.8), and ASD in siblings (RR=27) supporting a strong genetic contribution in the aetiology of autism (Lauritsen, Pedersen, and Mortensen 2005).

Combining information from different family studies Szatmari estimated the recurrence rate in siblings of affected children to be 1.1-3.3% (95% confidence interval) while the risk for any PDD was estimated to be between 1.6-5.6% (Szatmari et al. 1998). While not a high number in absolute terms it is very high compared to the 5-6 per 10,000 prevalence rate in the general population at the time, but yet much lower than risk in single-gene disorders.

1.2.1 Molecular genetics

The molecular genetic of autism is a fast moving field, with new results reported frequently. Several common variants have been associated with autism in genome-wide association studies (Pearson & Manolio 2008), most recently a region of chromosome 5 (5p14.1) (Wang et al. 2009; Ma et al. 2009). In 2011 it was suggested that at least 103 genes and 44 genomic loci are associated with ASD or AD. These genes and loci have all been causally implicated in intellectual disability as well (Betancur 2011). In addition to the common variants rare genomic alternations have also been linked to autism (e.g 1q21.1) (Mefford et al. 2008). Of particular interest for neurodevelopmental disorders are de-novo mutations. De-Novo mutations are alterations in a gene that are present for the first time in one family member as a result of a mutation in a germ cell (egg or sperm) of one of the parents or in the fertilized egg itself (De Rycke et al. 2002) . An association between de-novo mutations and autism has been reported (Neale et al. 2012) . However, despite the high heritability of autism, and new discoveries, the molecular basis of autism is still elusive and there is sufficient evidence for a role for non-genetic and/or environmental mechanism (Engel & Daniels 2011)

1.2.2 Maternal age

Advanced maternal age is one of the most frequently studied risk factors for autism (Durkin et al. 2008; Larsson et al. 2005; Glasson et al. 2004; Maimburg & Vaeth 2006; Reichenberg et al. 2006; Hultman et al. 2011; Sasanfar et al. 2010; Grether et al. 2009; Burstyn et al. 2010). Despite the considerable amount of research of the relation between maternal age and autism, including epidemiological samples, study results are mixed: positive associations (Grether et al. 2009) and null associations (Hultman et al. 2011) have been reported in similar numbers, and therefore the presence of the associations is still strongly disputed (Reichenberg et al. 2010). Different studies have also used different study designs, differences in case-ascertainment (Grether et al. 2009; Reichenberg et al. 2006; Zhang et al. 2010; Hultman et al. 2011) and differences in availability and adjustment for confounding, adding to the difficulty of comparing them.

The goal of this thesis is to rigorously examine the association between maternal age and ASD; rule out potential confounding factors, and characterize the aetiological role of other maternal related factors.

1.2.3 Other risk factors

The following section describes potential confounding and modifying factors for the association between maternal age and ASD. This thesis will consider the potential effects of such factors as part of the analytic approach.

1.2.4 Paternal age

There is now strong epidemiological support for an increased risk of autism among offspring of older fathers (Grether et al. 2009; Hultman et al. 2011; Reichenberg et al. 2006). These studies cover several geographic regions and health systems. Other psychiatric disorders such as schizophrenia (Frans et al. 2011; Malaspina et al. 2001) and bipolar disease (Frans et al. 2008) have also been associated with advancing paternal age. Where Reichenberg and colleagues (Reichenberg et al. 2006) were the first epidemiological study to point out de-novo mutations as the possible underlying mechanism for paternal age effects in autism later biological studies have verified this as a likely mechanism (Sanders et al. 2012; Neale et al. 2012; O'Roak et al. 2012).

However, there are other mechanisms that can not entirely be ruled out as independent risk factors. One such mechanism can be the inheritance of traits which are associated with a delayed fatherhood. It has been particularly difficult to separate paternal from maternal age effects, and to study alternative mechanisms. This thesis is uniquely positioned to address those challenges.

1.2.5 Sex

In the aetiology of autism gender is the strongest known predictor for autism. ASD prevalence in the US among 8 year old children was 4.7 times higher in boys (1 in 54) than in girls (1 in 252) (Sunderam et al. 2009). The ratio may however differ. It may be as low as 1:1 for severe cases with de-novo mutations and as high as 1:11 for Asperger disorder(Gillberg et al. 2006). It has also been debated if the expression is the same for both sexes or different (Andersson et al. 2013; Hartley & Sikora 2009; Robinson et al. 2013) and if the aetiology is the same (Zachor et al. 2013) but with no strong support to favour sex differences in aetiology.

1.2.6 Pre- and perinatal factors

Several pre- and perinatal factors have been associated with autism in epidemiological studies , e.g. gestational age, birth weight, hypoxia (Apgar score, foetal distress, Caesarean section, and bleeding during pregnancy) (Hultman et al. 2002; Kolevzon et al. 2007; Losh et al. 2012). Obstetric conditions could be part of a causal pathway to autism risk and are therefore important to consider.

1.2.7 In-vitro Fertilization

The number of babies born worldwide through IVF in 2002 was estimated to range between 219,000 and 246,000 (de Mouzon et al. 2009). Approximately 1% of U.S. infants born in 2006 were conceived through IVF. Rates increase dramatically in older mothers (>35) (Sunderam et al. 2009). IVF have been associated with increased risk for post-natal intensive care (Middelburg et al. 2008; Ericson et al. 2002; Allen et al. 2008; Sunderam et al. 2009), congenital malformations (Olson et al. 2005), Cerebral Palsy (Källén et al. 2005; Lidegaard et al. 2005), and increased risk for mild delays in development (Bowen et al. 1998a).

Angelman's syndrome is characterized by severe intellectual disability and lack of

speech, as well as abnormal motor behaviours, and symptoms that have considerable phenotypic overlap with autism. Two studies reported higher than expected rates of Angelman's syndrome following IVF (Cox et al. 2002; Ørstavik et al. 2003; Gosden et al. 2003). Higher rates of infertility problems have been reported in parents of autistic children (Funderburk et al. 1983). In contrast, Maimburg and Vaeth reported that children born following IVF had a lower risk of autism (Maimburg & Vaeth 2007). In a Danish study following all Danish birth 1995-2003 using the Danish IVF register (5.3% of all Danish births) there was no statistically significant increased risk of ASD when controlling for maternal age and other possible risk factors even though there were some indications of increased crude risk and in certain subgroups (Hvidtjørn et al. 2011)

1.2.8 Psychiatric disorders in relatives

Several psychiatric disorders are more common among relatives of individuals with autism (Micali et al. 2004; Bailey et al. 1998; Wolff et al. 1988; Jokiranta et al. 2013; Sullivan et al. 2012). There is however no evidence for an increased risk of psychiatric admission after the birth of the autistic child (Bolton et al. 1998). An epidemiological study from 2005 reported increased risk of schizophrenia-like psychosis, RR=3.4, and affective disorder, RR=2.9 in parents of autistic children (including Asperger and PDD-NOS) even when adjusting for maternal age and perinatal risk factors (Larsson et al. 2005). Another study showed a strong association between autistic children and autistic disorders or broader psychiatric history in mother and in siblings (Lauritsen et al. 2005; Jokiranta et al. 2013).

Besides an association with advancing maternal age on the risk of psychiatric disorders there are also reports on an increased risk for perinatal and adverse pregnancy complications among schizophrenic women (Bennedsen 1998; Bennedsen et al. 1999). Thus, it is possible that presence of psychiatric history in mothers (particularly schizophrenia) will be associated with both later age of conception, and increased rates of pregnancy and delivery complication, thus confounding the association between maternal age and autism.

1.2.9 Summary

Although the effort to find genes explaining the risk of autism has indicated more than 100 genes, these genes only explain a very small part of the underlying aetiology (Betancur 2011). Instead of common genes the genetic aetiology for autism seems complex involving common genes, as well as several loci occurring de-novo and of different types (copy-number-variants, deletion, insertions) and also possibly epigenetic origins.

Several factors studied in epidemiological samples have been associated with the risk of ASD. Similar to the genetic factors, while important, and most are more important than any single genetic factor, on a relative scale, these variables can not explain any major part of the aetiology. Of the risk factors mentioned in the previous sections, this thesis focuses on maternal age. Although previously studied, the inconsistent results, and the fact that maternal age represent a modifiable risk, makes it both intriguing and important to study rigorously.

2 Aims

Maternal factors have long been suggested as important for autism aetiology. Among such factors, maternal age is the most obvious to investigate, potentially harbouring multiple risk mechanisms. Therefore the aim of this thesis is to characterize potential pathways through which maternal age might be operating and more generally to study autism risk where maternal age may have an influence. It is important to study maternal age, together with factors potentially confounding this association, as a tool to suggest biological pathways. It can also have consequences for public health as the age of parenting has been, and keeps increasing in the Western world. This thesis performed the following four studies:

First, to address the heterogeneity of association between advancing maternal age and autism in earlier published research, I performed a meta analysis. This study meet the aim of this thesis by characterizing the shape and magnitude of the maternal age association, combined over multiple studies and by considering potential pathways for maternal age: offspring gender and the effect of confounding generally. Second, to meet the inherent limitations of a meta analysis, the independent and bivariate association of maternal and paternal age and ASD were analysed using a large

multinational population cohort, including separately for AD and ASD and for male and female offspring separately. This study support the aim of the thesis by directly evaluating and, as a result, suggesting a “new” pathway through which maternal age can operate: differences in age between the parents at the time of birth of the offspring. The study further support the study aim by characterizing (describing the functional form of the maternal age and of maternal age in relation to the age of the spouse.

The two last studies study autism risk where maternal age may have an influence by considering infertility problems and by considering familial risk. Thus, in study three, infertility problems and different in-vitro fertilization (IVF) treatments were examined as a second potential source underlying the maternal age-autism association and to suggest biological pathways to autism. In different studies intellectual disability has been showed to have genetic overlap (Betancur 2011) with autism and have some overlap in phenotypic features. The risk for autistic disorder and intellectual disability after IVF were studied while addressing confounding and mediating effect of parental age, psychiatric history and pre-term birth. Male and female offspring were be considered separately. This study support the aim of the thesis by characterizing potential pathways through which maternal age may operate: factors associated with fertility problems (since fertility problem is increasing with advancing maternal age) and IVF treatment (since maternal age could potentially confound an association between different IVF treatments and autism - in any direction).

Finally, in the fourth study the importance of genetic versus non-genetic, environmental factors in the aetiology of autism was examined by estimating the family clustering of ASD. The relative familial recurrence risk as well as the heritability were estimated using a range of familial relations from twins to cousins. The analyses include gender comparisons and comparisons separately for AD and ASD. This study support the aim of the thesis by including maternal age as one of the variables confounding the within-family risk. If maternal age is associated with autism risk then advancing maternal age for two consecutive siblings may create or inflate a sibling recurrence risk. Not taking maternal age into account can potentially result in risk associated with maternal age being mistaken for an additive genetic risk.

2.1 Specific Hypotheses

The following a-priori hypotheses were tested:

A Maternal age and ASD

1. Advancing maternal age is associated with increased risk of ASD in the offspring.
2. Advancing maternal age and advancing paternal age are independent risk factors for ASD in the offspring.

B I*n-Vitro Fertilization (IVF) and ASD*

1. IVF treatments are associated with increased risk for autism and intellectual disability in the offspring.

C General aetiology of ASD. The following competing hypotheses were examined:

1. Genetic factors will contribute substantially to individual differences in liability to ASD.
2. Shared environmental factors will contribute substantially to individual differences in liability to ASD.

3 Methods

It is the scope of this section to describe important methodological problems when considerations the studies presented and how we have addressed these problems.

3.1 Design issues, biases and confounding

Incomplete or inaccurate ascertainment of outcome and improper selection of controls are causes of (selection) bias in observational studies. In clinical trials this is controlled for by randomizing subjects to exposure (treatment group) where the bias is resolved into variation that is then taken care of by the statistical model. For observational studies randomization is not an option why other techniques must be used.

As a way to address case ascertainment, besides being of interest by its own right, in the studies presented here, we have included AD instead of, or together with, the ASD diagnosis since the stricter ICD/DSM criteria for AD should be more specific and

thereby less affected by misclassification compared with the wider diagnosis of ASD. This approach was possible given the high power in our studies.

Since we, first, have had access to entire national yearly birth cohorts and, secondly, the follow-up for autism is done in a publicly financed and utilized health system with equal access, under the assumption of no or negligible confounding, we minimize the risk of selection bias inherit in sampling design such as case-control, case-cohort or self-selection of participants. The study on IVF treatments involve a particular selection problem. Where most earlier studies have compared “any IVF treatment” against spontaneous conceived children we have chosen, primarily, another approach by comparing IVF treatments among children born IVF only. A comparison between children born after a certain IVF treatment and spontaneous born children can introduce bias since a specific IVF treatment can not be separated from the underlying fertility factors.

Bias could also be introduced through temporal trends in the ascertainment of autism. For this purpose we have addressed include birth year and age at diagnosis in all our analyses and addressed the issue of calendar effects in sensitivity analyses.

Another potential source of bias is measurement errors, differential or non-differential. In our studies the primary variables of exposure are maternal age, paternal age and family relations (siblings, cousins...) and secondary exposure variables include offspring year of birth and attained age. While the measurement of these variables may be subject to errors in retrospective studies (Lyall et al. 2012) the use of national prospective population register avoid these problems.

Since neurodevelopmental disorders are considered highly genetic of origin we have included parental psychiatric history at the birth of the offspring as a confounder in the analyses. Psychiatric diagnoses are not generally available in Sweden before 1973 we are probably missing psychiatric history for some parents. Since this is a relatively rare event in the general population the lack of data is a smaller problem than the specificity of the diagnostic codes used. For this we rely on the validation studies done (Ekholm et al. 2005; Kristjansson et al. 1987; Ludvigsson et al. 2011) .

In our studies of family risk and of IVF the detailed data including date of offspring

diagnosis has allowed us to model the probability of autism using survival analysis (time-to-event) techniques by fitting poisson or Cox regression. These models adjust properly for differences in length of follow-up between different (sets of) individuals which otherwise can bias the results. For the model estimating the heritability we adjusted for the differences in length of follow-up by including parameters for birth year. In **study II** the date of diagnosis was available only for Sweden and Denmark. In this study we first included parameters for birth year in a logistic regression model including all sites. Then, as a sensitivity analysis, we applied survival analysis (stratified Cox regression) using Sweden and Denmark only using attained age as time scale and stratifying for birth year (Korn et al. 1997).

3.2 Meta analysis versus individual level data

In **study I** we address the role of maternal age in the risk of autism using the approach of meta analysis. Meta analysis methods offer techniques to assist when combining results from different studies (D F Stroup et al. 2000) . The meta analysis is most important to do when there is heterogeneity and inconsistencies in the different studies. By relating the study outcome to a variable available from several studies, outcome = b + b*X, a meta-regression can give better understanding of study differences.

A drawback of the meta analysis approach is the retrospective nature since all analyses has been made already and adoption has to be made to this. Not all studies can be combined if too different categorizations have been applied or if a continuous covariate approach have been used in some studies and a categorical data approach in others. Sources that add on to the heterogeneity between studies in the meta analysis, e.g. different confounding factors, can instead be addressed directly. This include for instance using the same covariates across all sites and, using same reference group. In this way the **study II** complement and extend the results from the meta analysis. Also, for our particular application, several of the data sources underlying the meta analysis are the same as the data used in the **study I**.

3.3 Family clustering

Family clustering of diseases can occur between generation, parent-child, within

generations, between siblings or cousins. Here we are considering clustering within-generations only. To measure the risk on an individual level due to family clustering we calculated the relative recurrence risk defined as a risk ratio (relative risk) for the probability of being diagnosed with autism conditional on an autism diagnosis in a family member. For autism, this has only been done reliably in one earlier study, a Danish study (Grønborg et al. 2013). We did this for pairs of monozygotic twins, dizygotic twins, full siblings, maternal half siblings, paternal half siblings and cousins. Where other studies calculated absolute recurrence risk conditional on autism in an **older** sibling we relaxed this model and calculated the recurrence risk using time-varying exposure and allow younger sibling to expose and older sibling as well which give us higher power. A potential problem in calculating recurrence risk is the dependence on prevalence. For increasing prevalences a later born child will always have higher risk than earlier born which could bias the estimates. We adjusted for this in our analysis in several ways (1) Including birth year as confounder, even if increase in prevalence can be driven by calendar time birth year will work as a surrogate; (2) By not conditioning on exposure in older sibling we do not allow the design to increase the problem; (3) We calculated relative recurrence risk in sub-groups of birth cohorts to check and describe the size of the problem and (4) A monotone temporal trend could show up as a violation against the proportional hazards assumptions in the Cox regression model - we checked for this.

The family clustering can be caused by a combination of common genes and shared family environment. Any genetically mediated risk in siblings should be proportional to the genetic heritage shares (Sham 1997) . Thus, mono-zygotic twins share on the average 100% of their genetic risk where dizygotic twins and full-sibling only share 50%, half-siblings share 25% and cousins share 12.8% on the average. The proportion of the phenotype variance explained by genetic factors is termed heritability. We estimated the variance components for the probability of an autism diagnosis expressed as a generalized mixed effect model (Rabe-Hesketh et al. 2008; Rijsdijk & Sham 2002).

Where the recurrence risk measure familial clustering of autism with interpretation on an individual level the heritability measure the simultaneous information from a wider

set of relatives to measure the amount of variance (differences) in the data that can be attributed to genetic sources and have an interpretation on the population level.

Heritability calculations have traditionally relied on twin studies which often leads to sample size problems due to the low rate of twinning, especially for rare diseases. We are extending the population of the Swedish twin register with family data to obtain higher power, improve the convergence of the models and allow more complete complex variance component models to be fitted. The standard twin model separate the genetic variance component into two components. The *additive genetic variance component* capture the main effect of alleles on the phenotype, transmitted from parent to child. The *dominance genetic component* is the result of interactions between alleles at single loci and is estimated only from relatives who share a genotype identical by descent (from the same parental allele), i.e. from full siblings and twins. Using the combined data from twins and families allow a model including all four variance components (the ACDE model) to be identified and estimated, $var(y_{ij}) = \sigma_A^2 + \sigma_D^2 + \sigma_S^2 + \sigma_E^2$ where the standard twin model which do not include information on twins reared apart only allow ACE and ADE to be identified and estimated (Rabe-Hesketh et al. 2008).

3.4 Statistical analysis

Since different subjects are followed for different length of time they will contribute with different amount of information to the study. To adjust for this, in **studies II, III and IV**, we apply survival analysis to study the association between exposure and autism. In **study IV** and **study II** we use Cox regression and in **study III** we use poisson regression. Poisson regression is commonly used in survival analysis and gives approximately the same parameter estimates as the Cox regression and allow the same relative risk to be estimated (Whitehead 1980) but facilitate inclusion of more than one time scale and allow the rate and risk versus time development to be estimated directly.

Many epidemiological studies chose to group continuous data into categories. In **study II** and **study III**, for a more efficient use of the true continuous scale we are using splines (Hastie et al. 2009; Smith 1979) where an outcome is described by an arbitrary flexible form, Y = s(x), without the need to specify any shape. This allow us to predict

the functional form of the relation. When applied to confounding covariates, e.g. for paternal age when studying maternal age, this approach can generally be expected to adjust better for confounding than when creating categories of the data (Benedetti & Abrahamowicz 2004). Poisson regression using splines can be described by a generalized linear model.

In **study II** we are fitting splines to a bivariate exposure, Probability(Autism) = s(maternal age, paternal age). To overcome the problem with a too restricted shape (too flat and lack of local smoothness), that is the case when using two independent spline functions, Probability(Autism) = s(maternal age) + s(paternal age), we have fitted thin-plate splines (Wood 2003). The thin-plate spline is a smoother in two-dimensions where the curvature is estimated in local circular regions moving over the bivariate, i.e. two-dimensional, surface similar to moving averages in the one-dimensional space. As far as we know this technique has never been used in epidemiological research on autism.

When there are several members from the same family contributing there may be problem with correlations in the data. While not biasing the point estimates the variances may be biased - in any direction. For this purpose we calculated robust standard errors using Generalized Estimating Equations (GEE) in **study IV and study III** using family or set of siblings or cousins as cluster. Another feature of the GEE is that the estimation do not require the data to follow any particular parametric distribution such as the poisson (Fitzmaurice et al. 2004, pp.291–320; Liang & Zeger 1986).

Throughout all four studies we have made extensive use of graphical methods. The purpose has been to offer a visually summary of complex results. Such results can sometimes be difficult to derive in tables and important messages might even get lost. For instance, we have chosen to plot relative risk estimates and the associated confidence intervals on a relative scale. Using tables it is not immediately obvious that a confidence limit 0.00781 should be given the same interpretation of precision as 128, since 0.00781 = 1/128 and complex relations between different relative risk estimates are easily spotted (Devesa et al. 1995). Similarly we present figures of the functional form of probability(autism) = s(maternal age) with a potential for a complex dose-response relation easily recorded by the eye.

In **study II** the data delivered from Israel included all autistic patients in Israel together with a random sample of population based controls, not the entire population as for the other sites. To adjust for this we applied inverse probability weights in the calculations with weights proportional to the sampling probability. Furthermore, as the usual variances in the statistical models do not correct for the variation (insecurity) of the sampling weights we used robust standard errors. The precision is only slightly decreased by this approach.

In **study IV** we calculated relative recurrence risk using survival analysis. In this analysis, by definition, only pairs of family members contribute. In the analysis a sibling (or cousin) pair enter the cohort when a second sibling (cousin) is born . From that date both members contribute with information as unexposed (X=0). If one member is being diagnosed with autism the exposure data this individual contribute with is then changed to exposed (X=1) and the follow-up continues until the other member is also being diagnosed with autism or end of follow-up what ever comes first. Both members in the pair enter the analysis exposing each other. As far as we know this approach has not been published earlier and will contribute with higher power compared with analysis only allowing an older sibling to expose a younger sibling. Yet other studies have used logistic regression methods using matched cohort design (Lichtenstein, Björk, et al. 2006; Svensson et al. 2012) with lower power and with the risk of introducing biases due to differences in length of follow-up.

3.5 Missing data

Missing data can be an important source for bias or reducing power in epidemiological studies. There are different approaches to address these problems such as Multiple Imputation (Rubin & Schenker 1991) or Multiple Imputation by Chained Equations (White et al. 2011). However, if only a relatively small proportion of the data contain missing values the records containing missing data can safely be deleted using only the complete cases in the analysis.

Moreover, in the statistical literature missing data are usually classified according to the underlying reason of being missing. For data missing completely at random (MCAR), the probability of a value being missing is independent of both the observed data and the unobserved data, e.g. by tossing a dice, comparisons are generally not subject to

bias. When, in a function Y = f(X) relating an outcome with exposure X, the probability of a particular value Y being missing depends only on the observed data (Y or X), then the missing data are said to be missing at random (MAR). If the missing data can be considered missing at random, the estimates obtained from the maximum likelihood estimation, such as poisson regression or logistic regression, are unbiased. If this assumption is not true, the missing data are not ignorable and the missing mechanisms should be modelled (Little & Rubin 1987).

In the work presented here using registers the data at hand are essentially complete. For instance, in the IVF study, only 0.7% had missing information on paternal age, maternal age or pre-term status. Similar figures apply for the other studies. These small numbers of missing data will not be able to bias the results or to affect the precision in any meaningful way. We also believe the statistical methods applied should be robust for data being missing due to any foreseeable reasons.

3.6 Data sources

Except for the meta analysis all studies utilized register based data for autism and exposure. In study 2 and 3 we used Swedish register data and in study 4 we used Swedish register data combined with register data from Denmark, Finland, Norway, Israel and Western Australia in the iCARE collaboration (Schendel et al. 2013). We describe the Swedish data sources and comment on the iCARE data separately.

3.6.1 The basis for register based research in the Nordic countries

All health care in the Nordic countries (Sweden, Denmark, Finland, Norway and Iceland), is publicly financiered and utilized. Especially for psychiatric diagnoses where no private clinics exist the population coverage is close to complete (Ludvigsson et al. 2011). Furthermore, all Nordic countries are utilizing unique personal registration number in all contact with authorities (as well as private organizations and companies). The identifiers are automatically assigned each citizen at birth or at immigration. The health system, publicly financed and utilized, avoid selection biases otherwise often present in the creation of epidemiological cohorts (Delgado-Rodríguez & Llorca 2004) and the personal identifiers allow information from different services to be joined on an individual level. This set-up create a unique environment for epidemiological

research (Allebeck 2009; Olsen 2011).

There is a huge variety of registers at use for administrative and research purposes. Below the registers used in our studies are described in more detail. The health registers; the Medical birth register, the Patient register and the IVF register; are controlled and managed by the National Board of Health and Welfare ("Socialstyrelsen"), a government agency in Sweden under the Ministry of Health and Social Affairs.

3.6.2 Medical birth register

The Medical Birth Register (MBR) was established in 1973 (Axelsson 2003). It contains data on pregnancy and birth for all births in Sweden. More than 95% of the Swedish pregnant population attend antenatal care before the 15th gestational week and the register covers over 98% of births in Sweden from 1973 and onwards (Anon 2003). During the first visit, usually during pregnancy week 8-13, the woman is asked about the number of years of involuntary infertility. The information is recorded in the MBR. The register includes information collected prospectively, starting with the first antenatal visit through the time when mother and child are discharged from the hospital after delivery. All birth reported to the MBR are validated each year by cross-checking the personal registration number against the Register of the Total Population. Antenatal care routines are standardized and the information is provided through antenatal, obstetrical, and neonatal records, and classified according to the International Classification of Diseases (ICD) version 8 until 1986, version 9 from 1987 to 1996, and version 10 subsequently. The register include information about still birth, multiple birth, gestational age, birth weight, sex and Apgar score.

3.6.3 Population vital statistics

Individual vital statistics data including date of birth, date of emigration, date of immigration and date of death is maintained by Statistics Sweden (Total Population Register, Emigration and immigration register).

3.6.4 Multi-generation register

The Multi-generation register contains information about the entire Swedish population (Ekbom 2011; Anon n.d.). The register has been extensively used for

different research purposes. Children born from 1932 and alive 1961 are linked to their biological parents. In 1991 all records were computerized for all persons registered 30th June 1991 while persons deceased between 1947 and 30th June 1991 were not computerized. In several later waves the persons missing in the register 1991 have been added in. The register comprises about 10 million children (index persons, 2011) together with their biological parents. Importantly the register includes family information (e.g., identification of parents, siblings and offspring) allowing linkage to other population based registers, which include information on health (e.g. psychiatric hospitalizations), demographic variables (e.g., date of birth, death and emigration).

3.6.5 Patient register

Sweden has universal and publicly financed health insurance coverage that guarantees equal access to health services, regardless of employment status, socio-economic status or regional residency. The register has a nationwide coverage of patient treatment facilities and includes care in psychiatric as well as somatic hospitals. There are no private psychiatric hospitals in Sweden. The Swedish National Patient Register contains details on virtually all new psychiatric hospitalizations since 1973 (Ludvigsson et al. 2011). Before 1973 there is data for selected counties only. The register include data on admission and discharge dates and the discharge diagnosis made by the treating physician. Besides this diagnosis, the main diagnosis, there are also secondary diagnoses. Outpatient visits are included since 1999 but only from specialist care, thereby excluding any diagnosis by general practician. Diagnostic information is coded using the ICD codes. The standard procedure dictates that diagnosis will be given by a consultant (equivalent of an attending) psychiatrist at the time of discharge from hospital. The diagnostic assessment is then forwarded on a computer medium to the National Patient Register. These routines are standardized across Sweden.

All infants and preschool children are regularly seen at well-child care clinics and undergo routine medical and developmental screening. All children aged 4 undergo routine general health screening, that includes mandatory developmental assessment (motor, language, cognitive and social development) conducted by a nurse and paediatrician. Children with any suspected developmental disorder (including autistic disorder and intellectual disability) are referred for further assessment by a specialized

team in a child psychiatry unit or habilitation service. During the study period diagnoses were made by diagnostic teams with a psychiatrist, clinical psychologist, and speech pathologist or occupational therapist, depending on clinical manifestations. The instruments include parental interviews, cognitive testing of the child, and observations in naturalistic settings, including the home or the unit. The Patient Register contains the diagnostic information. The Patient-Register has shown high reliability for somatic and psychiatric diagnoses (Kristjansson et al. 1987; Ludvigsson et al. 2011). For a diagnosis of intellectual disability the evaluation is made by a psychologist and according to standardized tests with high reliability.

3.6.6 IVF register

The IVF register contain frequencies of all IVF/ICSI treatments in Sweden from 1982 to 2007. Since 2003 data on embryos transferred are registered as well. From 2007 data are stored in a separate Swedish "quality register" including a higher degree of details.

The 16 clinics for IVF/ICSI Sweden are required by law to report all treatments. IVF/ICSI treatments are offered to women in the range 25-42 years of age. There are no strict age restrictions for males. Eligibility requires a medically documented fertility problem. In Sweden, almost exclusively, IVF is used to treat female infertility while ICSI is used for male infertility.

3.7 The iCARE data base - A multinational data source

The International Collaboration for Autism Registry Epidemiology (iCARE) is a multinational collaboration across (the Nordic countries), Denmark, Norway, Finland and Sweden and Israel and Western Australia combining population based register date from all countries. As a result a database combining exposure data such as parental age, pre-term birth, gestational age, parity, apgar score with autism diagnosis has been developed. The design is a cohort consisting of all children born in the contributing countries between 1987 (since no diagnosis of autism earlier) and 2004 with follow-up until 2009. In total the combined database contain 36,736 persons with ASD ascertained from 6,8 million children.

The database include selected variables from the Swedish Medical birth register, Register for vital statistics and patient register, all described above, and the

corresponding registers in the five other contributing countries. The iCARE project and the created autism cohort has been extensively described (Schendel et al. 2013).

Table 1 Summary of iCARE data sources

Site	Million Births (1987-2004)	ASD cases 1987-2009	AD cases 1987-2009
Denmark	1.05	10,338	2,910
Finland	1.02	7,388	1,440
Israel	1.46	2,091	-
Norway	1.04	1,284	652
Sweden	1.82	14,501	5,386
W. Australia*	0.41	1,134	810
TOTAL	6.80	36,736	11,198

* A state based register containing both state and private register data. Follow-up to 2004.

A special problem in the iCARE project has been the ethical and legal issues raised by national authorities when using national health data for research on an individual level. In iCARE we have solved this by setting up a federative database solution (Muilu et al. 2007) where data physically stays on local servers in each country and anonymous data are transferred to the analysis server (in Australia) for each new analysis and erased after the execution.

3.8 Diagnostic coding system

The Swedish patient register is recording diagnoses from the International Statistical Classification of Diseases (ICD). Different version have been in use starting; ICD-7 1964-68, ICD-8 1969-86, ICD-9 1987-96 and ICD-10 from 1997. Only from the ICD-9, year 1987 is there a diagnosis available for autism. Cases of autism before 1987 were most likely coded as Schizophrenia with additional description of “childhood”. The National Board of Health and Welfare (“Socialstyrelsen”) is obtaining code translations between the different coding systems on their web page (http://www.socialstyrelsen.se/english). The ICD system is also used by the health registers in Denmark, Finland and Norway.

The ICD-10 and DSM IV criteria for ASD are almost identical.

3.9 Power considerations

The power of a statistical test is the probability of rejecting the null hypothesis when the null hypothesis is false. The power is a concept useful for planning purposes and only relevant before any data have been collected (Hoenig & Heisey 2001; Levine & Ensom 2001). It is especially important to consider in the study of rare outcomes such as autism (and rare exposures).

First, for **study I**, we are combining all available and relevant studies in a meta-analysis. Meta-analysis is a tool in itself to address possible power issues in possibly under-powered studies.

For the analysis of maternal age in the iCARE collaboration, **study II**, the plan was to have at least 6,000 cases of AD and 23,000 cases ASD - in the end we obtain twice as many cases. The Swedish prevalence of infantile autism for children born after 1987 is about 0.4% and, including a wider spectrum, for ASD about 0.9%. In the planning phase the power was calculated by arguments:

1. Assuming 25% and 10% of the birth having parents 25-30 yrs and >40 yrs and with 0.02% prevalence of AD there will be 90% power to detect an odds-ratio of 1.2 or bigger in Sweden alone.
2. Assuming 25% and 10% of the birth having parents 25-30 yrs and >40 yrs and with 0.02% prevalence of AD there will be 90% power to detect an odds-ratio of 1.1 or bigger.

In Sweden there are about 100,000 birth each year resulting in a total of 2 million births between 1987-2007, with about 325,000 women >= 35. When planing the **study III** we assumed 4% IVF among these women (since more common in older mothers) there are about 13,000 IVF women to be compared with about 310,000 non-treated women. If the underlying autism prevalence among untreated is 0.4% and if the true (but unknown) relative risk is at least 1.5 we have a power of at least > 87% to detect this difference using a simple chi-2 test on the two-sided 5% level of significance. Please note that the calculations above must be considered conservative. Using more efficient statistical models for survival to adjust for actual follow-up time and including covariates to adjust for important confounding the power will be even higher.

Table 2 Power calculations assuming 310,000 subjects in the control group and N (1,000) in the treated group (in the table)

Reference % Diseased	Relative Risk	N	Power (%)	Reference % Diseased	Relative Risk	N	Power (%)
0.05	1.50	6.5	6.84	**0.60**	1.50	6.5	76.03
	1.50	13.0	13.07		1.50	13.0	97.38
	1.75	6.5	10.88		1.75	6.5	98.02
	1.75	13.0	24.64		1.75	13.0	99.99
	2.00	6.5	16.79		2.00	6.5	99.97
	2.00	13.0	40.52		2.00	13.0	100
	3.00	6.5	57.75		3.00	6.5	100
	3.00	13.0	94.37		3.00	13.0	100
0.4	1.50	6.5	55.48				
	1.50	13.0	87.41				
	1.75	6.5	88.60				
	1.75	13.0	99.66				
	2.00	6.5	98.87				
	2.00	13.0	100				
	3.00	6.5	100				
	3.00	13.0	100.00				

For **study IV** we estimated recurrence risk and heritability using the entire Swedish population - all data available.

3.10 Statistical software

We used different statistical software. As the standard work horse horse for data management the SAS software was used (http://www.sas.com/). The SAS software version 9.2 and 9.3 was used for all four studies presented here. Also, most statistical graphs were produced using the SAS/GRAPH software.

The glimmix procedure in the SAS/STAT software version 9.3 was used for the Poisson regression models in the IVF study. Except for the thin plate spline regression, the glimmix and genmod procedures in the SAS/STAT software version 9.3 was used for the statistical models in the multinational parental age study. Both glimmix and genmod

was used for the GEE estimation technique. The SAS/STAT power procedure was used for power calculations when planning the studies.

For the thin-plate regression in the multinational study on parental age the R software (R Core Team 2013) 2.15.2 (2012-10-26), package ***gamm4 version 0.1-6*** (Wood & Scheipl 2013, p.4) was used. This statistical model was not available in the SAS software.

For the Cox regression, with robust standard errors, fitted to estimate the relative recurrence risk in the study on family risk the coxph function in the R software, package ***survival (Therneau 2013)***, was used on a Red-Hat linux server. This function was considerably faster than corresponding procedure in SAS, procedure phreg. However, the SAS phreg procedure was used for the calculations using the iCARE database.

The figures showing the relative risk estimates was produced using SAS/GRAPH. In the same study the OpenMX software (Boker et al. 2011) was used to calculate the heritability in autism.

For the meta-analysis of maternal age and the risk of ASD the R package ***metafor*** (Viechtbauer 2013) was used.

This thesis is written using the open source word processor LibreOffice 4.0 (http://www.libreoffice.org) (LibreOffice Documentation Team 2013). The references were managed using the open source Zotero software (http://www.zotero.org/). Where not mentioned above, all software were run on the Linux Debian sid operating system (http://www.aptosid.com).

3.10.1 Federative database solutions in iCARE

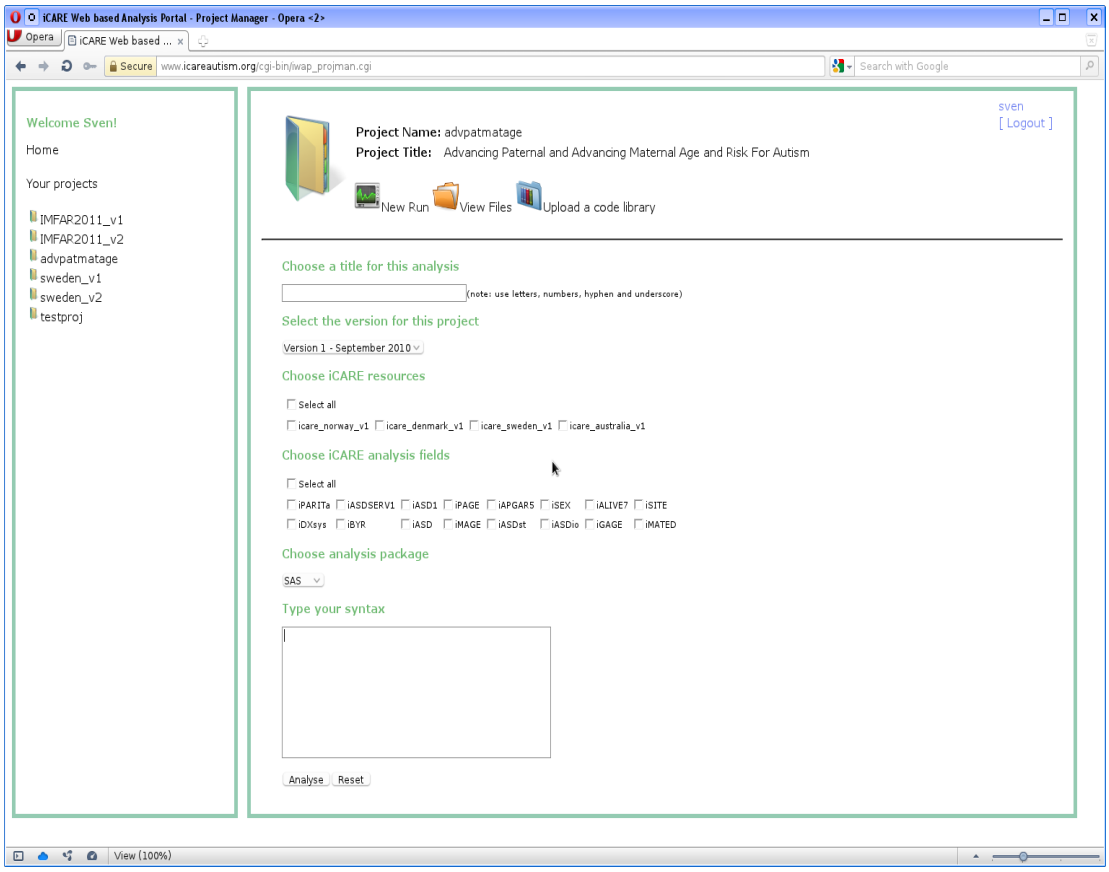
The iCARE data base, section 3.7, include individual data. Not all countries allow such data to leave the border of the country for storage and pooling with other data. As a solution to this the federative database solution has been created (Haas et al. 2002). A federative database is unique in that data from different sites (countries, companies, database solutions, storage formats etc.) are accessed and combined without a common storage, or even data standard, and in a secure way ensuring the protection of the data at each site.

I made a considerable contribution in developing the solution for working with the

federative database. For the solution within ICARE open source software was used as far as possible. Each site administer a server where the data is stored. The server can be a physical computer or a virtual server residing within another server. Each site server store the site data in a MySQL database. To access the data the ICARE analysis server must be used. This is physically located in Perth, West Australia, and managed by the collaborators responsible for IT at the Telethon Institute for Child Health Research. Access to the data and the server is restricted by security protocols and signature of the analysis server (by IP-address).

For analysing the ICARE data a web browser is used. Access to the analysis site (https://www.icareautism.org) is only possible if a security protocol with unique user id and password has been installed. The protocol and password is managed by the collaboration IT in Perth and last for one year at a time. Once connected the data can be analysed by uploading computer code (SAS software, STATA software or the R software) which is submitted in batch mode. Results are stored on the analysis server and can be downloaded when ready. For this purpose a web interface facilitating the analysis process has been developed (**Figure 2**).

Figure 2 The ICARE web interface for data analysis



4 Study I - Maternal age in autism - A meta analysis

This section contain a study published in Journal of The American Academy of Child and Adolescent Psychiatry. Accepted 24 February 2012. published online 06 April 2012.

Biography: Sandin S, Hultman CM, Kolevzon A, Gross R, Maccabe JH, Reichenberg A. Advancing maternal age is associated with increasing risk for autism: a review and meta-analysis. J Am Acad Child Adolesc Psychiatry. 2012 May;51(5):477–486.e1.

DOI: 10.1016/j.jaac.2012.02.018

Note: This document differ only slightly from the submitted manuscript.

1. When updating the reference list for the viva the reference to R software , number 36 disappeared. I replaced this with a more current reference (R Core Team 2013).
2. The references 31 and 41 in the published manuscript referred to the same manuscript. Here there is one reference only.

4.1 Summary of the study

Objective

We conducted a meta-analysis of epidemiological studies investigating the association between maternal age and autism.

Method

Using recommended guidelines for performing meta-analyses, we systematically selected, and extracted results from, epidemiological scientific studies reported before January 2012. We calculated pooled risk estimates comparing categories of advancing maternal age with and without adjusting for possible confounding factors. We investigated the influence of gender ratio among cases, ratio of infantile autism to autism spectrum disorder (ASD), and median year of diagnosis as effect moderators in mixed-effect meta-regression.

Results

We found 16 epidemiological papers fulfilling the a priori search criteria. The meta-analysis included 25,687 ASD cases and 8,655,576 control subjects. Comparing mothers ≥35 years with mothers 25 to 29 years old, the crude relative risk (RR) for autism in the offspring was 1.52 (95% confidence interval [CI] = 1.12–1.92). Comparing mothers ≥35 with mothers 25 to 29, the adjusted relative risk (RR) for autism in the offspring was 1.31 (95% CI = 1.19-1.45). For mothers <20 compared with mothers 25 to 29 years old, there was a statistically significant decrease in risk (RR = 0.76; 95% confidence interval = 0.60–0.97). Almost all studies showed a dose-response effect of maternal age on risk of autism. The meta-regression suggested a stronger maternal age effect in the studies with more male offspring and for children diagnosed in later years.

Conclusions

The results of this meta-analysis support an association between advancing maternal age and risk of autism. The RR increased monotonically with increasing maternal age. The association persisted after the effects of paternal age and other potential confounders had been considered, supporting an independent relation between higher maternal age and autism.

4.2 Introduction

Most plausible neurodevelopmental theories of autism focus on genetic factors (Bailey et al. 1995). However, there is evidence that non-heritable, pre-, or perinatal events, and/or environmental exposures are likely to also have a significant etiological role (Bristol et al. 1996).

Advanced maternal age is one of the most frequently studied risk factors for autism (Durkin et al. 2008; Larsson et al. 2005; Glasson et al. 2004; Maimburg & Vaeth 2006; Croen et al. 2007; Reichenberg et al. 2006; Daniels et al. 2008; Shelton et al. 2010; Grether et al. 2009; Lundström et al. 2010; Lauritsen et al. 2005; Croen et al. 2002; Eaton et al. 2001; Fombonne 2005; Burstyn et al. 2010; Williams et al. 2008; Windham et al. 2011; Leonard et al. 2011). However, the results from the individual studies are mixed and the presence of the associations is still disputed (Reichenberg et al. 2010).

It is important to examine the relationship between advanced maternal age and autism for two main reasons. First, an association between maternal age and autism may provide clues to the biological pathways leading to autism. Older maternal age has been associated with increased rates of chromosomal abnormalities (Salem Yaniv et al. 2011). Older mothers also have increased risk of obstetric complications possibly due to uterine muscle dysfunction and diminished blood supply with age (Bolton et al. 1997; Kolevzon et al. 2007). Cumulative exposure to environmental toxins may also be important for the association between advanced maternal age and neurological and psychiatric disorders (Lundström et al. 2010).

Second, age of parenting has been increasing in the United States and Europe in recent decades (Bray et al. 2006; Martin et al. 2006) and an association between maternal age and autism may help explain the increase in prevalence estimates of autism during the past two decades.

In order to elucidate the association between advanced maternal age and autism we conducted a systematic review and meta analysis of all population-based epidemiological studies published until June 2011 which investigated the association between advancing maternal age and autism. We also explored possible sources of heterogeneity across studies.

4.3 Methods

The meta-analysis was based on recommended guidelines (Donna F. Stroup et al. 2000; Lau et al. 1997; Stangl & Berry 2000).

Data sources

We identified published peer-reviewed studies through search of PUBMED using the keywords "autism" together with "maternal" or "paternal" or "parental" or "obstetric" or "perinatal" together with the words "risk" or "association" or "associated". We only included papers published in English between 1 Jan 1990 to 31 December 2011. We screened the resulting abstracts and obtained full text versions of potentially relevant studies. We then hand-searched the reference lists of original articles to identify any missing papers.

Table 1: List of studies and study characteristics identified for meta analysis

									Adjustment for confounding					
Studies Author, country, publication year	**Diagnostic method**	**Birth years**	**Median year of diagnosis**	**% AD**	**M/F sex ratio**	**Design**	**Cases**	**Non-cases**	**Birth order**	**Birth year**	**SES**	**Pre-natal**	**Psych hist.**	**Ethnicity**
Durkin, US 10 states, 2008	DSM-IV	1994	1998	80.7	4.5	Case-cohort	1,251	253,347	X	X	X	X		
Larsson, DK, 2004	ICD 8/10	1973-99	1986	NK	3.2	NCC	698	17,450	X	X	X	X	X	
Glasson, Australia, 2010	DSM-III / IV	1980-95	1989.5	68	5.3	CC	465	1,313	X	X		X		
Maimburg, DK, 2006	ICD 8/10	1990-99	1994.5	100	4.1	NCC	473	4,730		X		X		X
Croen, US, 2007	ICD 9	1995-99	1999.5	47	5.4	Cohort	593	132,251	X	X	X			X
Reichenberg, Israel, 2006	ICD 10	1980-85	1983	>90	5.5	Cohort	110	132,161		X	X			
Hultman, SWE, 2010	ICD 9/10	1983-92	1994.5	100	3.2	Cohort	860	1,034,627	X	X	X	X	X	X
Sasanfar, Iran, 2010	DSM-IV	1994-2001	2005	68	3.8	Case-cohort	179	549,354	X	X	X			
Grether, US, 2009	DSM III/IV	1989-2002	1997.5	NK	4.9	Cohort	20,701	6,506,555	X	X	X	X		X
Lundstrom, SWE, 2010	DSM-IV	1992-98	1995	NK	NK	Twin Cohort	164	10,884	X	X	X			
Lundstrom, UK, 2010	DSM-IV	1994-96	2005	57	5.4	Twin Cohort	193	12,904				X		

CC: Case-control, NCC: Nested case-control, AD: Infantile autism, SES: Socio economic status, NK: Not known. Note: All studies adjusted for paternal age and sex. Study 8 additionally adjusted for smoking during pregnancy. The five right most columns for confounding represent model covariates for Birth order, Socio economic status (Paternal and/or maternal education, Source of payment of delivery), Prenatal (Gestational age, Weight for gestational age, Birth weight, Foetal distress, Apgar score, Congenital malformations, Foetal position), Psychiatric history (Maternal and/or paternal psychiatric history), Ethnicity (Maternal/Paternal race or country of origin). Study (Lundström et al. 2010) included two parts, one Swedish and one UK. For the UK part we received additional data from the authors. Study (Grether et al. 2009) utilizing the California Department of Development Services did not distinguish between autistic disorder and autism spectrum disorders since a service registry was used.

Study selection

We used the following inclusion criteria: (1) a population based sample of cases using one of two of the major clinical diagnosis systems, DSM or ICD (**Table 1**) ; (2) comparison subjects drawn from the general population with information on parental age obtained from the same source; (3) use of a format for presentation of data allowing for comparisons between studies and calculation of relative risk measures; (4) presentation of results for maternal age and (5) adjustment for paternal age. The standard of reporting associations for maternal age in the autism literature is using age-band categories.

Data extraction

The following information was extracted from each study: estimates of relative risk (odds-ratios from case-control or cohort studies or incidence rate ratios or hazard ratios from cohort studies) separate from crude and multi-variable adjusted models, study design, number of ASD cases and non-ASD controls, confounding covariates used in adjusted model(s), year of diagnosis, birth cohort, diagnostic method, ratio of autistic disorder and autism spectrum disorder cases, male to female ratio among the autism cases, and how maternal and paternal age was modelled, e.g. categorically. These data are summarised in **Table 1**.

Additional data

When necessary, authors were contacted and additional information was requested.

Statistical methods

We calculated weighted relative risk (RR) estimates and associated two-sided 95% confidence intervals (CIs). Computations utilized the published RR and CI values assuming approximately normal distribution. Extensive research has demonstrated that age 35 is the age at which risk for a range of adverse developmental outcomes (e.g., Down's syndrome) increases and therefore younger ages are typically used as a reference points. Since best supported by the available studies the primary comparison contrasted maternal age group 25-29 years with maternal age group ≥35 or >40 years.

To examine if there is an increasing risk with increasing maternal age and the potential risk associated with younger maternal age we also contrasted maternal age group 25-29 years with mothers <20 years and with mothers 30-34 years.

When modelling the log(RR) we allowed for both a within-study variance of the log relative risk and for a between study variance term assuming the data to follow a normal distribution. With y_i indicating the log(RR) extracted from the publications the random-effects models can be defined as $y_i = \mu + u_i + e_i$ where $u_i \sim N(0,\tau^2)$ denotes the normal distributed between-study variation and $e_i \sim N(0,\sigma^2)$ denotes the normal distributed within-study variation. The statistical model accommodates crude and adjusted RR estimates to be included. From the published papers we extracted both *crude* models including a categorical covariate for maternal age only and adjusted models including and adjusting for possible confounding effects as well. Models were fitted separately for the crude and multivariable adjusted estimates and separately for the different category comparisons, e.g. ages 25-29 vs ≥35. Robustness of results was evaluated by (a) excluding the study with the largest effect size, and (b) excluding the study with the largest sample.

Potential publication bias was examined using funnel plots (Iyengar 1988) and by calculating Egger's test (Egger et al. 1997). The funnel plot shows the effect size of the different studies on the x-axis and an estimate of the sample size on the y-axis. Small studies should have higher variability in estimates of relative risk compared with bigger studies while divergence from this pattern may indicate the presence of publication bias.

Potential sources for study heterogeneity were examined using meta regression analysis. Using the above model this was done by replacing the term u_i with $\beta_0 + \beta_1 x_{i2} + \beta_2 x_{i2}$ where the parameters β_1 and β_2 measure the size of the association of the moderators in a mixed-effects model. The mixed-effects models were fitted by a maximum likelihood technique that allows for model comparisons using the Akaike's Information Criteria (AIC) (Daniels et al. 2008) for which a lower AIC value indicate better model fit. The proportion of males among cases and the proportion of autistic disorder among the cases were examined. Also, since the rate of autism has been increasing we included a covariate allowing for a fix change of exposure effect across

calendar time in a supplementary model to reduced the between study heterogeneity. For descriptive purposes RR estimates were calculated by levels of the moderating variables on RR estimates of maternal age are presented by median levels of the moderating variables.

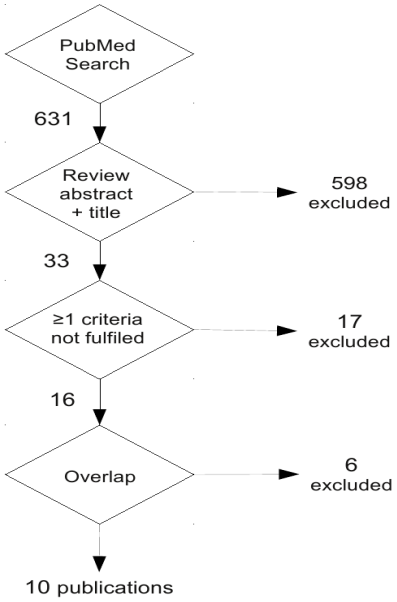
Data was analyzed using R statistical software version 2.12.1 with the Metafor package ver 1.4.0 (Viechtbauer 2010; Anon 2005) and SAS ver 9.22 procedure GLIMMIX. Statistical significance level was set at two-sided 5% level corresponding to two-sided 95% confidence intervals of the pooled relative risk estimates.

4.4 Results

4.4.1 Overview of study characteristics

Our search criteria resulted in 631 published papers. 598 studies were excluded after an initial review of the titles and abstracts carried by two of the authors (SS and AR). The remaining 34 studies were carefully reviewed and 17 were further excluded (see **Figure S1**).

Figure S1 Flow chart with numbers showing published papers selected and excluded from the initial search in PubMed to the publications included in the final pooling and meta-analysis. Note: Overlap indicates a published paper in which the study population overlap with another study already included in the meta-analysis



Eleven studies, from the US (Durkin et al. 2008; Croen et al. 2007; Grether et al. 2009), Denmark (Larsson et al. 2005; Maimburg & Vaeth 2006), Australia (Glasson et al. 2004), Israel (Reichenberg et al. 2006), Sweden (Lundström et al. 2010; Hultman et al. 2011), Iran (Sasanfar et al. 2010) and the UK (Lundström et al. 2010)[b] fulfilled all 5 inclusion criteria and were included in the meta analysis (**Table 1**). The two Danish (Larsson et al. 2005; Maimburg & Vaeth 2006) used nested case-control designs drawn from the national total populations. The study from Western Australia (Glasson et al. 2004) was a population based case-control design with the entire population of Western Australia as reference population, and the study from Iran (Sasanfar et al. 2010) a case-cohort design drawn from a cohort of pre-school children aged 4-11 years. The three studies from the US, the Israel study (Reichenberg et al. 2006) and the most recent Swedish study (Lundström et al. 2010) all used population based cohort designs while studies (Lundström et al. 2010 a; Lundström et al. 2010 b) were cohort studies on Swedish and UK twins.

Six other studies were excluded from the meta-analysis mainly due to overlap; Two studies from Sweden were excluded (Daniels et al. 2008; Hultman et al. 2002) because they overlap with a later study (Lundström et al. 2010), and, because of concern for under-ascertainment of autism cases due to changes in autism services in Sweden in one of the studies (Daniels et al. 2008 p. 1360). Two studies from the US (Shelton et al. 2010; Windham et al. 2011) were not included because of substantial overlap with another study (Grether et al. 2009) which examined a considerably larger cohort. Another US study (Bilder et al. 2009) was also excluded due to overlap with the two earlier studies and did not meet the initial requirement of clear presentations of the results for the risk associated with maternal age with only crude estimates available and a different categorization of maternal age (<20, 20-34, >34). A study (Leonard et al. 2011) was excluded due to substantial overlap with study (Glasson et al. 2004), use of case prevalence instead of case incidence, and sub-dividing the cases into children with and without intellectual disability. Two Danish studies (Lauritsen et al. 2005; Hvidtjørn et al. 2011) were not included in the formal pooling due to overlap with the other two Danish studies and lack of adjustment for paternal age as only crude estimates were available.

Covariates used for adjustment for possible confounding in each study are specified in **Table 1**. All studies included in the meta-analysis adjusted for paternal age, birth year and sex. All but two studies (Glasson et al. 2004; Maimburg & Vaeth 2006) were adjusted for SES; all but three studies (Croen et al. 2007; Reichenberg et al. 2006; Sasanfar et al. 2010) for obstetric condition (e.g., apgar score, being small for gestational age, birth weight); all but five (Larsson et al. 2005; Glasson et al. 2004; Reichenberg et al. 2006; Lundström et al. 2010; Sasanfar et al. 2010) for parental ethnicity. Only two studies (Larsson et al. 2005; Lundström et al. 2010) adjusted for parental psychiatric history.

4.4.2 Meta-analyses

The primary meta-analysis was conducted on the association between maternal age and ASD. The 11 studies included in the analysis had a total of 25,687 ASD cases and 8,655,576 subjects without an ASD diagnosis.

The crude results showed statistically significant support for an increased risk of autism in the offspring of mothers aged 35 or older compared with mothers aged 25-29 in 8 of the 11 studies (**Table 2**). The random-effect pooled estimate of the *crude* risk of autism in mothers aged 35 or older compared with mothers aged 25-29 years was 1.52 (95% CI: 1.21-1.92), p-value<0.001.

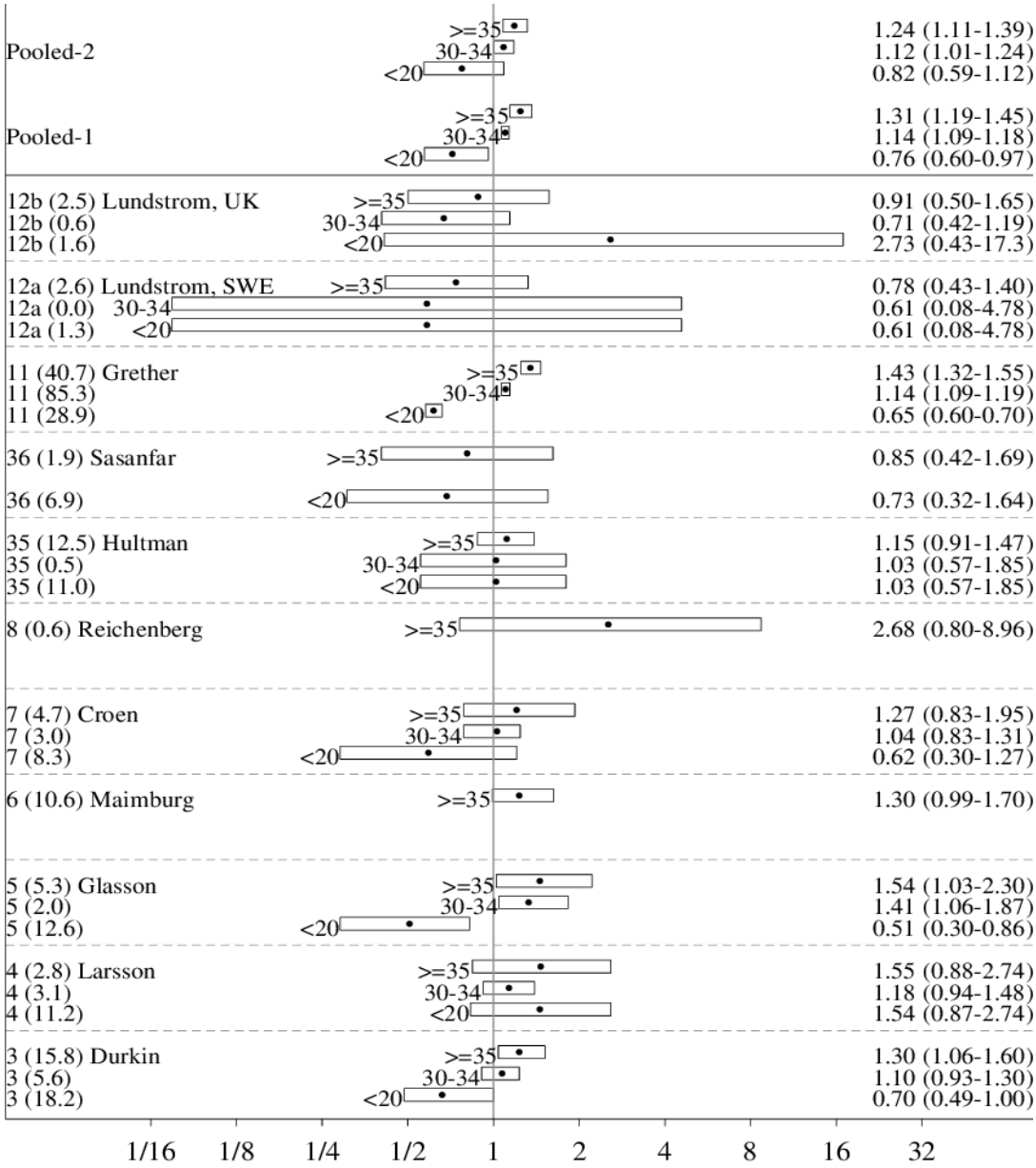
Table 2 Relative risk (RR) point estimates and two-sided 95% confidence intervals comparing 25-29 year old mothers with mothers ≥ 35 or ≥ 40 adjusting for potentially confounding covariates. Relative study weights in the pooling procedure.

Study	Crude RR	Crude 95% Confidence Limits	Adjusted RR	Adjusted 95% Confidence Limits	Weights	Weights*
(Durkin et al. 2008)	1.38	1.17-1.64	1.30	1.06-1.60	16	30
(Larsson et al. 2005), #	2.19	1.36-3.52	1.55	0.88-2.74	3	4
(Glasson et al. 2004)	NA	NA	1.54	1.03-2.30	5	8
(Maimburg & Vaeth 2006)	1.60	1.28-2.00	1.30	0.99-1.70	11	18
(Croen et al. 2007), #	1.53	1.05-2.24	1.27	0.83-1.95	5	7
(Reichenberg et al. 2006)	9.68	3.51-26.7	2.68	0.80-8.96	1	1
(Hultman et al. 2011), #	1.53	1.26-1.86	1.15	0.91-1.47	12	22
(Sasanfar et al. 2010)	1.17	0.69-1.99	0.85	0.42-1.69	2	3
(Grether et al. 2009), #	1.84	1.72-1.97	1.43	1.32-1.55	41	*
(Lundström et al. 2010 a), Swe	1.01	0.67-1.52	0.78	0.43-1.40	3	4
(Lundström et al. 2010 b), UK	0.78	0.51-1.20	0.91	0.50-1.65	3	4
Pooled I	1.52	1.21-1.92	1.31	1.19-1.45		
Pooled II*	1.59	1.25-2.03	1.24	1.11-1.39		

*) Excluding the study by Grether et al (Grether et al. 2009)
#) 25-29 year old vs. ≥ 40 (all other 25-29 year old vs. ≥ 35)

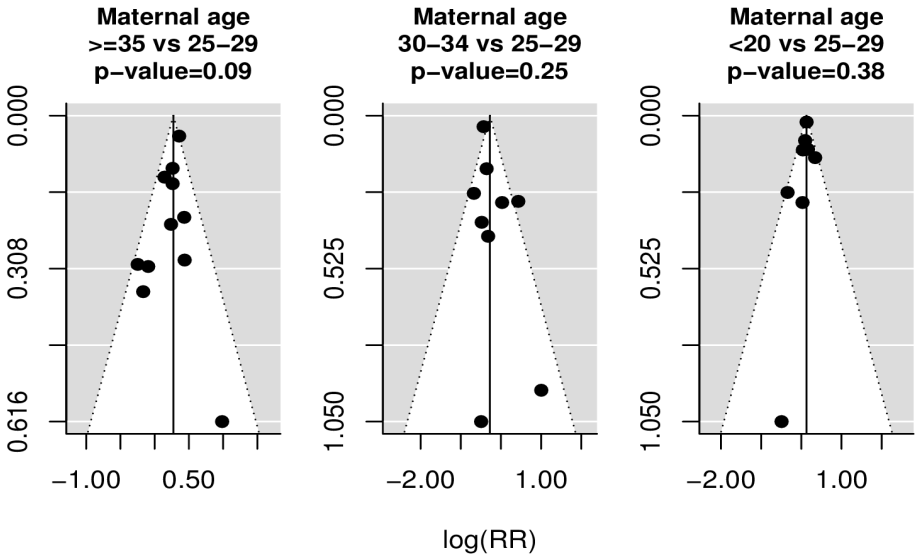
The crude associations were reduced in all studies after adjustment for potentially confounding covariates (**Table 2**). Associations nevertheless remained statistically significant in 3 of the studies (Durkin et al. 2008; Larsson et al. 2005; Glasson et al. 2004). After adjustment for potential confounding covariates the random-effect pooled estimate of risk of autism in mothers aged 35 or older compared with mothers aged 25-29 years was 1.31 (95% CI: 1.19-1.45) (**Figure 1**), p-value<0.001.

Figure 1 Association between increasing categories of maternal age and risk for autism spectrum disorder (ASD). Presented are the adjusted relative risk (RR) comparing 25- to 29-year-old mothers with younger (<20) and older (30 –34 or ≥35 years) mothers. Note: RR on the x-axis. Black dots and horizontal bars outline relative risk point estimates and associated two-sided 95% confidence intervals for the relative risk of autism spectrum disorder in offspring comparing mothers <20, 30 to 34, and ≥35 years (bottom to top within each study) years with mothers 25 to 29 years. Study number to the far left. Pooled results on the upper part of the figure where pooled-2 do not include one study (Grether et al. 2009) in the calculations. Exact numbers for RR and the confidence intervals to the right. In parentheses to the right of the study number the study weight (value 0 –100) in the pooling procedure is shown.



There was no evidence to support publication bias (**Figure S2**), and the test of heterogeneity between studies was not statistically significant

Figure S2 Funnel plots. Note: Standard error vs. log(relative risk (RR)) corresponding to RR estimates in Table 2. P values corresponding to Egger's test of publication bias



Because the study from California (Grether et al. 2009) which showed a statistically significant association between advancing maternal age and ASD contributed as much as 20,701 of the ASD cases to the meta analysis this study was excluded in a sensitivity analysis. The pooled results were similar even after this study was excluded.

When the association between maternal age and autism was examined across the range of categories of maternal age there was evidence for a monotonic increase in risk of autism with increasing maternal age categories. Of the 9 studies which included more than one age group comparison all but two studies (Larsson et al. 2005; Lundström et al. 2010 b) reported findings that were consistent with a monotone maternal age effect. **Figure 1** shows the associations between increasing categories of maternal age and risk of ASD in the offspring. The effect was only minimally attenuated after excluding the study from California (Grether et al. 2009).

In a complementary analysis we also examined the studies reporting RR for maternal age ≥40(Larsson et al. 2005; Croen et al. 2007; Grether et al. 2009; Lundström et al. 2010) and the studies only reporting on maternal age ≥35 (Durkin et al. 2008; Glasson et al. 2004; Maimburg & Vaeth 2006; Reichenberg et al. 2006; Lundström et al. 2010; Lundström et al. 2010; Sasanfar et al. 2010) separately and compared the RR for the

highest age category. The RR for maternal age ≥40 compared with maternal age 25-29 was 1.37 (95% CI: 1.19-1.58) and the RR for maternal age a ≥35 compared with maternal age 25-29 was 1.23 (95% CI: 1.09-1.39) (p-values<0.001).

Combining studies (Durkin et al. 2008; Larsson et al. 2005; Glasson et al. 2004; Croen et al. 2007; Grether et al. 2009; Sasanfar et al. 2010; Lundström et al. 2010) to evaluate the risk associated with younger maternal age (<20) with 25-29 years old mothers showed a statistically significant decrease in risk (RR: 0.76; 95% CI: 0.60-0.97, p-value=0.028). Excluding the highly influential study (Grether et al. 2009) the RR point estimate was slightly higher but now the confidence interval included 1.0 (RR: 0.82; 95% CI: 0.59- 1.12).

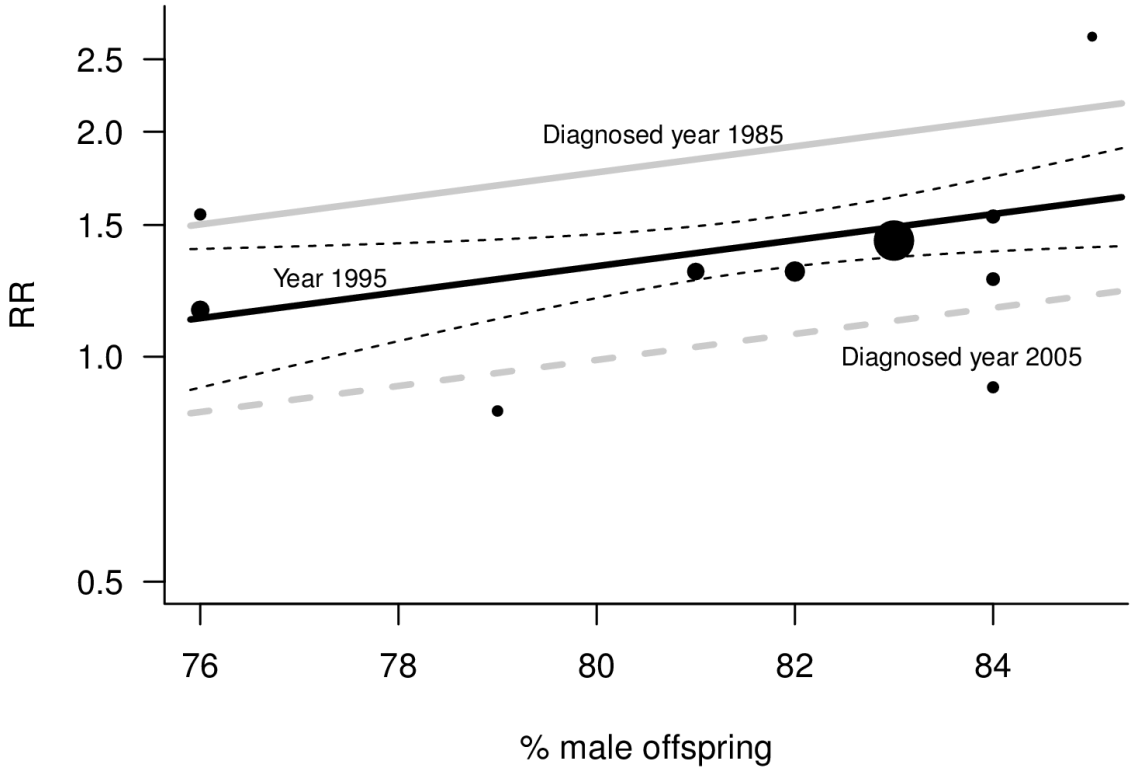
4.4.3 Moderator analysis and meta-regression

Meta-regression was used to assess if the effect of maternal age on the risk of autism was modified by other study-specific covariates. Three variables were considered as potential moderators in the meta-regression analyses: Percent male offspring in the study,; study year of autism diagnosis (median of first and last diagnosis) and percent ASD cases diagnosed as autistic disorder. Information about study year of autism diagnosis was available in all 11 studies (Durkin et al. 2008; Larsson et al. 2005; Glasson et al. 2004; Maimburg & Vaeth 2006; Croen et al. 2007; Reichenberg et al. 2006; Grether et al. 2009; Lundström et al. 2010 a; Lundström et al. 2010 b; Sasanfar et al. 2010; Hultman et al. 2011). Information on percent male offspring was available in ten, and information on percent with autistic disorder was available in eight studies.

For mothers ≥ 35 the covariates percent male offspring and year of autism diagnosis, were both statistically significant when controlling for each other jointly. Year of diagnosis was statistically significant among mothers 30-34 when simultaneously adjusting for percent male offspring or percent autistic disorder. For maternal age <20 the percent male offspring was statistically significant when entered as a single variable and also when adjusting for year of diagnosis. To summarise: For all three age categories of maternal age a higher amount of boy offspring strengthened the effect of maternal age; positive for maternal ages 30-34 and ≥ 35 and negative for maternal age < 20 while the maternal age effect diminished with later year of diagnosis. The moderating effect of the percent male offspring and year of diagnosis among mothers

≥ 35 is summarised in **figure 2**.

Figure 2 Predicted relative risk of autism (RR) for maternal age ≥35 vs. maternal age 25 to 29 years as a function of percent male offspring and year of diagnosis. Note: Each dot indicates the RR for each study. Solid line and 95% confidence limits predicting association for diagnosis in 1995. Gray solid and dashed lines for predictions 1985 and 2005, respectively



For ease of interpretation and to quantify the impact of the three potential moderators RR estimates were calculated in subgroups of these variables (**table 3**). The increasing effect with increasing maternal age remain in all sub-groups.

Table 3 Relative risk (RR) and associated two-sided 95% confidence intervals (CI) comparing maternal age 25-29 years with maternal ages <20, 30-34 and ≥ 35 years in sub-groups of the moderator variables

	< 20 years			30-34 years			≥ 35 years		
	N	**RR**	**95% CI**	**N**	**RR**	**95% CI**	**N**	**RR**	**95% CI**
Sub-groups by percent male offspring*									
≤ 82%	4	0.93	0.64-1.37	3	1.12	0.98-1.28	5	1.25	1.10-1.42
> 82%	4	0.65	0.60-0.70	4	1.14	1.09-1.19	5	1.42	1.32-1.53
Sub-groups by year of diagnosis*									
≤ 1995	4	0.90	0.50-1.61	4	1.24	1.05-1.46	6	1.26	1.08-1.46
> 1995	5	0.65	0.61-0.70	4	1.13	1.09-1.18	5	1.34#	1.19-1.51
Sub-groups by percent infantile autism*									
< 74%	4	0.62	0.43-0.89	3	1.07	0.76-1.50	4	1.22	0.94-1.58
≥ 74%	2	0.79	0.56-1.12	2	1.09	0.93-1.29	4	1.26	1.10-1.45

N: Number of studies in each sub-group, * median across studies (82.5%, 1995 and 74%), # In the sub-groups of year of diagnosis for mothers ≥ 35 one study (Grether et al. 2009) had a substantial impact on the >1995 sub-group potentially accounting for the high RR in this group .

4.4.4 Additional analyses

Advancing maternal age has been associated with increased risk for obstetric complications (Lundström et al. 2010), and several obstetric conditions have been associated with increased risk for autism (Kolevzon et al. 2007). Six studies in the meta-analysis also controlled for the effects of obstetric conditions (**Table 1**). After adjustment for obstetric conditions the association between advancing maternal age and autism remained statistically significant in three (Durkin et al. 2008; Larsson et al. 2005; Grether et al. 2009) of the six studies (Durkin et al. 2008; Larsson et al. 2005; Glasson et al. 2004; Maimburg & Vaeth 2006; Lundström et al. 2010; Lundström et al.

2010; Grether et al. 2009) with RR for maternal age ≥35 compared with maternal age 25-29 estimated at 1.41 (CI: 1.31-1.52) and 1.37 (CI: 1.27-1.49) respectively (p-values <0.001); (**Table 2**). Two of the studies not included in the meta analysis also reported a statistically significant association between autism and older age of mothers after adjustment for obstetric complications (Burstyn et al. 2010; Williams et al. 2008; Lundström et al. 2010). The RR of autism associated with a 10 year continuous linear increase in maternal age was available in 6 studies (Durkin et al. 2008; Croen et al. 2007; Reichenberg et al. 2006; Grether et al. 2009; Lundström et al. 2010; Hultman et al. 2011) with a pooled estimate RR of 1.23 (95% CI: 1.19-1.27) and when excluding the study (Grether et al. 2009) RR: 1.07 (95% CI: 0.99-1.15).

4.5 Discussion

The role of advancing maternal age in the aetiology of autism has been debated (King et al. 2009; Baxter et al. 2007). This meta analysis supports the assertion that advancing maternal age at the time of birth is associated with an increasing risk for autism and spectrum disorders. The association between advancing maternal age and risk of autism in the offspring was robust to adjustment for confounding including paternal age, obstetric complications, birth year, birth order and markers for socio-economic status, with offspring of mothers older than 35 having 30% increased risk for developing autism.

There was some support that the association between maternal age and autism varied as a function of the proportion of male cases and year of diagnosis. A stronger association between maternal age and risk of autism was observed in studies with a higher proportion of male offspring cases. These results are not conclusive, but are intriguing nevertheless. Two previous studies (Croen et al. 2007; Reichenberg et al. 2006) observed that the effects of paternal and maternal age on risk of autism varied as a function of the offspring sex. The association between advancing paternal age and autism was stronger in female offspring, whereas the association between advancing maternal age and autism was stronger in male offspring. The moderator analysis supports this hypothesis which may point to possible sex-specific aetiology in autism.

Year of diagnosis was another potentially moderating variable suggesting that the effect of advancing maternal age may have been decreasing over time. Year of

diagnosis was also noted as diluting the effect of maternal age in an earlier study (Grether et al. 2009). A possible explanation includes age dependent changes in ascertainment of autism and autism spectrum disorders, or changes related to changes in the risk or ascertainment of phenotypic subtypes (Grether et al. 2009).

Although previously reported (Croen et al. 2007), and speculated as a cause for the moderating effect of year of diagnosis (Grether et al. 2009), the association between maternal age and autism did not vary between different autism sub types in the meta-regression.

These results, like those of any meta-analysis, should be viewed with caution. Meta-regression is a form of observational association and therefore cannot be used to make causal inferences about the data (Higgins & Thompson 2004). There may be confounding factors that underlie the relationships reported here. In addition, given the differences between studies in covariates selected and availability, the meta-regression analysis captured only some of the studies included in the meta analysis. Despite these potential shortcomings, our results suggest that research on maternal age and autism should consider the effects of potential moderating factors. Another potential issue for this article is the largest study included in the meta-analysis[11], contributing 80% of the number of cases. Inclusion of this study could be considered problematic also because autism cases were ascertained only if they had both a diagnosis of autism as well as a substantial functional impairment (Grether et al. 2009). However, our sensitivity analysis demonstrated that inclusion of this study did not bias the results of the meta analysis or the moderator analysis. Removing this study from the analysis did not substantially change the magnitude of the association of the meta analysis, and the effect of year of diagnosis and proportion of males remained statistically significant. A potential limitation of the study is that access to the data was restricted to categories of maternal age. This did not allow exploring the full underlying maternal age continuum. Finally, in our focus on published epidemiological studies we do recognize there may also be other important aspects that would have required inclusion of more clinically oriented papers but in the context of the meta analysis may be less reliable.

Potential aetiological mechanisms of maternal age

One possible explanation for the maternal age effect is an increased occurrence of genomic alterations. Numerous neurological and psychiatric disorders have been related to genomic alterations (Reichenberg et al. 2009). Maternal age is an important factor in the aetiology of chromosome anomalies (Ginsburg et al. 2000; Martin 2008) and genomic modifications (Kaytor et al. 1997; Orr & Zoghbi 2007). Interestingly, a number of studies have uncovered an increased prevalence of de-novo copy-number variants (CNVs), and other forms of genomic alterations, in autistic children (Christian et al. 2008; Marshall et al. 2008; Sebat et al. 2007), supporting the notion that novel mutational events may be important in the pathogenesis of autism. Whether these events are also related to advancing maternal age remains to be determined.

An alternative explanation is that epigenetic dysfunction underlies some parental age effects. 'Epigenetics' refers to the heritable, but reversible, regulation of gene expression (Henikoff & Matzke 1997). Epigenetic dysfunction has been associated with several neuropsychiatric disorders (Mill et al. 2008), and is also implicated in single-gene disorders, including Rett's and Fragile X syndromes, characterized by autistic-like features in some patients(Reichenberg et al. 2009).

It is also possible that the accumulated exposure to various environmental toxins over the life-course could result in genomic and/or epigenetic alterations in the germ cells of older parents. Toxins have been shown to induce DNA damage, germline mutations and global hypermethylation (Yauk et al. 2008) in germ cells, and have long term developmental consequences in offspring (Williams & Ross 2007). In addition, increasing maternal age may be related to endocrine and hormonal factors, not only by ageing alone but also through maternal stress, increasing infertility and assisted reproductive treatment (Newschaffer et al. 2007).

In conclusion, this meta analysis supports an association between advancing maternal age and risk of autism. The relative risk increased monotonically with increasing maternal age. The association persisted after the effects of paternal age and other potential confounders have been considered, supporting an independent relation between higher maternal age and autism.

5 Study II - Parental age in a large multinational cohort

This section contain the manuscript as submitted for a last round with the co-authors.

5.1 Summary of the study

Background

Autism spectrum disorders (ASD) are developmental disorders affecting 1-2% of all children. Advancing paternal and maternal age have been suggested as risk factors for autism, but although there are several population-based studies, results are inconclusive. This study examines whether advancing paternal and advancing maternal age are independently associated with risk for ASD

Methods

This study was a population based cohort study from five countries (Denmark, Israel, Norway, Sweden and Western Australia) including all live born singletons at different periods from 1985 through 2004 . Information on parental ages, demographic characteristics, pregnancy and ASD outcome in the offspring were obtained from patients, service and medical registries. The study included all live born singletons. Relative risk (RR) was calculated using logistic regression and splines to link the probability of ASD with parental age continuously. For maternal and paternal age separately but also as a bivariate exposure. Graphical methods were implemented as analytic strategy to examine qualitative aspects of the data and to enhance interpretation.

Results

Among 5,766,794 singleton births in the study 30,474 (0.53%) had ASD. Advancing paternal age and advancing maternal age were each associated with increased risk for ASD in the offspring (p=0.0001) after controlling for potential confounders and the other parent's age. Yet, bivariate models showed that highest risk for ASD is seen in three groups approximated by (I) Fathers older than 45 independent of maternal age; (II) Fathers 35-45 with mothers ≥10 years younger; and (III) Mothers 30-40 years old with father ≥10 years younger. These groups included 7.2% of all births. We did not find support for a modifying effect of the sex of the offspring and results were similar,

although with greater effect sizes for autistic disorder. Younger maternal age (<20) was also associated with increased risk for ASD (p=0.0001).

Conclusions

Advancing paternal age and advancing maternal age at the time of birth of offspring increases the risk of ASD. De novo germline and somatic mutations may partly explain these associations. The evidence for risk associated with older parental age coupled with large parental age differences suggests that social factors, in particular assortative mating, play an important role as well.

5.2 Introduction

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by difficulties in social interaction and communication accompanied by stereotypic, repetitive behaviour and narrow interests (Hollander et al. 2010). Once believed to be rare, ASD now reportedly affect 1-2% of children in developed countries. Twin and family studies provide compelling evidence for a substantial role for genetic factors in the aetiology of autism (Bailey et al. 1995; Grønborg et al. 2013). However, studies also emphasize that environmental influences are aetiologically important (Hallmayer et al. 2011).

Advancing parental age has been repeatedly investigated in relation to ASD risk. Associations between older age of fathers as well as older age of mothers have been reported (Grether et al. 2009; Hultman et al. 2011; Sandin et al. 2012; Parner et al. 2012). Although there are several population-based studies, meta-analyses have shown considerable heterogenity between studies in the magnitude of associations (Hultman et al. 2011; Sandin et al. 2012), and it has been difficult to determine whether the effects of paternal and maternal age represent independent risk factors. This is important because the main reason to examine the relationship between parental age and ASD is that it provides clues to the biological pathways leading to autism, and different mechanisms may mediate advancing paternal or maternal age (Kolevzon et al. 2007).

The association between advancing parental age and ASD is be unclear due to several methodological limitations. These include variation in, or lack of, statistical control for other risk factors (e.g., age of the other parent, birth order, perinatal complications);

high levels of missing-data; selection bias; and differences in the classification of parental age categories. Furthermore, even population based studies have been limited by sample size and could not reliably examine the risk associated with parental age in tails of the age distributions or to properly separate the independent and combined effects of paternal and maternal age.

The goal of this study was to provide highly reliable estimates of the association between parental age and ASD, and determine the combined and independent effects of the paternal and maternal age on risk.

The study build on a unique resource: the "International Collaboration for Autism Registry Epidemiology (ICARE)" (Schendel et al. 2013), a multinational collaboration combining harmonized national cohorts across several geographic regions and health systems with the purpose of studying risk factors for autism.

5.3 Methods

This study uses a population-based cohort design based on data assembled by iCARE (Schendel et al. 2013). ICARE utilizes nation- or state-wide health and administrative registers from six countries, Denmark (DK), Finland, Israel (ISR), Norway (NOR), Sweden (SWE) and Western Australia (WAU) for the study of familial and environmental risk factors for autism. ICARE development, resources, data coordination, standardization and harmonization procedures have been described in detail (Schendel et al. 2013). Specific details relevant for this study are provided below. Ethical approval for the study was obtained from each iCARE member's ethical committee or Institutional Review Board (IRB).

5.3.1 Study population

The study population is comprised of all live born singletons in Denmark, Norway, Sweden between January 1st, 1985 and December 31st, 2004. Data from Western Australia were comprised of all 1985-1999 birth. Data from Israel were comprised of all 1993-2004 births with a subsequent ASD diagnosis and a random sample of controls during the same birth year period. All births were derived from comprehensive population data reported to each site's nation- or state wide medical birth register. Linkage of individual level data on all cohort members (birth, outcome, exposure and covariate information) was based on unique personal identification numbers available to each site for data preparation purposes (although no personal identification numbers are retained in any final ICARE harmonized dataset). Universal health care coverage that is publicly financed and utilized is available to all citizens of Denmark, Israel, Norway and Sweden and contacts with the public health care system are required to be reported. In Western Australia both public and private health care provisions are available although both public and private health care providers are required to report to the public health system. This means that for this study the cohort information from registries across all sites is not biased by differential access to health care and is population-based.

5.3.2 ASD Outcome, Parental Age and Covariate information

Cohort members were followed from birth through 2009 a reported diagnosis of an ASD in Denmark, Israel and Sweden, through 2006 in Norway and through 2004 in Western Australia. Two sites (Denmark, Sweden) retrieve autism diagnosis data from government-maintained medical registries that record diagnoses or procedures from each in- or outpatient clinic or hospital contact. For the other three sites (Israel,

Norway, Western Australia) diagnostic information is derived from government-maintained service/benefits registries that record contacts with individuals receiving services/benefits for autism. ASD diagnoses were based on ICD versions 8, 9 and 10 in Denmark, Norway and Sweden while the DSM-IV classification system was used in Israel and Western Australia. The iCARE harmonization protocol for ASD diagnostic codes across the different classification systems is provided in **Table S2**.

Parental ages, sex and year of birth of the offspring were obtained from the comprehensive birth or civil registration registries at all sites.

Table S2 Harmonization of autism spectrum disorder (ASD) diagnoses across different diagnostic systems

Diagnostic System and Associated Codes	ICD-8 • 299.00/01/02/03 (Psychosis; used in Denmark to indicate autism)	ICD-9 • 299.0 (AD) • 299.1 (Disintegrative psychosis) • 299.8 (Other) Note: Should include Asperger Syndrome and Other PDD) • 299.9 -unspecified Note: Should include PDD-NOS.	ICD-10 • F84.0 (AD) • F84.1x (Atypical Autism) • F84.5 (Asperger Syndrome) • F84.8 (Other PDD) • F84.9 (PDD-NOS) • F84.2 (Rett Syndrome)	DSM-IV • 299.0 (AD) • 299.1 (Childhood Disintegrative disorder (CDD)) • 299.8 (Rett Syndrome) • 299.8 (Asperger Syndrome) • 299.8 (PDD-NOS)
iCARE ASD Categories				
Autistic disorder (AD))	299.00	299.0	F84.0	299.0
Asperger Syndrome (ASP)	299.02	299.8	F84.5	299.8
Pervasive developmental disorder – not otherwise specified (PDD-NOS)	299.01, 299.03	299.9	F84.9 F84.1x, F84.8	299.8

When multiple diagnoses are available for a given individual: If ever Rett Syndrome or CDD, then classify as **not ASD**, regardless if also ever had an ASD diagnosis. Iif never Rett Syndrome or CDD, then classify as: (a) AD: Autistic disorder/childhood autism if ever received this diagnosis (i.e., disregard other ASD subtype diagnoses), (b) ASP: If never autistic disorder/childhood autism AND ever had Asperger syndrome (i.e., disregard other ASD subtypes), (c) PDD-NOS: if never AD and never ASP and ever (PDD-NOS OR ATYPICAL AUTISM OR OTHER PDD)

5.3.3 Statistical methods

The data were analysed by fitting logistic regression to the data relating the probability of autism to a linear function of the covariates. Since the data from Israel were obtained using a case-cohort design, with known sampling probabilities for the cases and controls, rather than a birth cohort design we included the sampling weights in the analyses (Borgan et al. 2000). Sampling weight equal to one was used for all non-Israel individuals. To adjust for the variation introduced by the weights robust standard errors were used (Barlow 1994). For the purpose of achieving a qualitative interpretation of the functional form of risk for ASD across all parental ages we fitted splines (Smith 1979; Hastie et al. 2009). Also, using splines for adjustment of a continuous variable allow for a more detailed adjustment for confounding than using categories of the same variable (Benedetti & Abrahamowicz 2004). The relative risk (RR) of autism by categories of parental age is presented in tables to quantify the degree of association. All tests of statistical hypotheses were done on the two-sided 5% level of significance.

The analyses of paternal and maternal age independently were done in the following steps; First, in a qualitative approach, we fitted splines to continuous maternal age (years) and paternal age (years) separately while adjusting for site, sex and confounding: the age of the other parent categorically (<20, 20-24, 25-29, 30-34, 35-39 and ≥40 for mothers and 40-44 and ≥45 for fathers) and birth year (4-year intervals). Second, to quantify the risk, RR was calculated by age category instead of using splines. To adjust for differences in the observed rate of autism all models allowed for different intercepts for each site and for male and female offspring separately. The relative risk of autism and associated two-sided 95% Wald type confidence intervals was calculated for each parental age.

In an analysis unique for the high power of the current dataset we also analysed paternal and maternal age jointly. This was done in two ways. **First** using thin-plate splines (Wood 2003) we created a 3D figure illustrating the risk of ASD as a function of maternal and paternal age jointly including interaction effects and adjusting for sex and birth year categorically as above. The thin-plate spline is a smoother in two-dimensions where the curvature is estimated in local regions moving over the bivariate, i.e. two-dimensional, surface similar to moving averages in the one-dimensional space. We calculated number of births and number of ASD and AD cases by categories of pairs of paternal-maternal ages. **Second**, to remove the effect of the spouse efficiently we analysed parental age in sub-groups of the spouse. To avoid the sub-group analysis being entirely driven by the number of autism cases as would be the case with hugely different number of autism cases in different sub-groups, we created subgroups of maternal age with approximately equal number of autism cases in each maternal age subgroup. This resulted in maternal age cut-offs ≤26, 27-31 and >31 years. For each subgroup independently we fitted the logistic spline models, crude and adjusted. We used the same approach for the analysis of maternal age in subgroups of paternal age (≤28, 29-34, >31). **Third**, to support the joint approach to the risk of autism we also fitted logistic regression models including separate covariates for mean age of mother and father and for the difference in age between mother and father. The first covariate capture the direct effect of ageing while the difference in age capture the effect not necessarily associated with ageing. To examine which of the two components is most important we compared goodness of fit including the two components by calculating the AIC.

5.3.4 Supplementary analyses

We calculated RR for offspring of male and female sex separately. In addition to the analyses for all ASD, we repeated the same set of analyses for autistic disorder only (not including Israel data where information on the diagnostic categories of ASD was not available).

A binomial model such as logistic regression assumetion that all subjects have been exposed to the same amount of risk time (equal length of follow-up) or that the length of follow-up does not affect the risk. If the assumption is not correct biases can be introduced. For this purpose we included birth year as a covariate in all our models. In addition to this we also performed a sensitivity analysis including Denmark and Sweden only, which had data on date of diagnosis. For Denmark and Sweden combined we fitted Cox regression and compared the estimated RR and associated confidence intervals with the corresponding results from logistic regression. Similarly, since (in a family) parental age is directly associated with calendar time bias from increasing prevalences by calendar time can be suspected. The fitted Cox regression model described above will adjust for calendar effects as well (Korn et al. 1997).

5.3.5 Statistical considerations

In studies combining data from different sources there may be an issue of site-to-site heterogeneity. With the big sample size at hand any unspecific test for differences in shape of parental age between sites is likely to be statistically significant. Instead, with the focus on qualitative differences rather than quantitative differences we addressed this by visual inspection of the graphs of the spline predictions and verifying combined results by additional models excluding any potentially heterogeneous site.

5.4 Data management

All data are stored, combined and analysed using the approach of secure database federation (Muilu et al. 2007; Haas et al. 2002). The statistical analyses were done using SAS software version 9.3. The thin-plate splines were fitted using the gamm4, ver 0.1-6 (Wood & Scheipl 2013, p.4) package running the R software version 2.15.2 (Anon n.d.) on a linux 64-bit server.

5.5 Results

The multinational combined ICARE cohort included a total of 5,766,794 births, 30,474 (0.53%) children with ASD and 10,082 (0.17%) children with AD. Distributions of births, cases, birth year and parental age by paternal and maternal age are presented in

Table 1.

Table 1 Covariates distributions by parental age

	Births (% Male)	ASD (%)*	AD (%)*	Maternal Age#	Paternal Age#	Birth Year
Maternal Age						
<20	128,017 (50)	684 (0.53)	207 (0.16)	18 (17-19)	23 (17-28)	1994 (1986-2002)
20-29	3,125,845 (51)	16,275 (0.52)	5,093 (0.16)	26 (22-29)	29 (24-35)	1994 (1987-2002)
30-39	2,374,617 (51)	13,007 (0.55)	4,485 (0.19)	33 (30-37)	35 (30-41)	1995 (1987-2003)
>=40	138,283 (50)	936 (0.68)	343 (0.25)	41 (40-43)	42 (35-49)	1996 (1988-2003)
Paternal Age						
<20	27,303 (53)	174 (0.64)	54 (0.20)	19 (17-22)	18 (17-19)	1994 (1986-2002)
20-29	2,156,605 (51)	10,963 (0.51)	3,298 (0.15)	25 (21-30)	26 (23-29)	1994 (1986-2002)
30-39	2,993,093 (51)	15,842 (0.53)	5,316 (0.18)	30 (25-36)	34 (30-38)	1995 (1987-2003)
40-49	540,562 (53)	3,497 (0.65)	1,288 (0.4)	35 (28-40)	43 (40-47)	1996 (1988-2003)
>=50	49,137 (51)	426 (0.87)	172 (0.35)	35 (28-42)	53 (50-58)	1996 (1988-2003)

*: Cases (percent), # mean (10th percentile, 90th percentile)

Table 2 Relative risk (RR) of autism spectrum disorder (ASD) and autistic disorder (AD) by age intervals of parental age

	Age Interval *	Maternal Age Crude	Maternal Age Adjusted #	Paternal Age Crude	Paternal Age Adjusted #
ASD	<20/20-29	1.22 (1.13-1.32)	1.22 (1.12-1.33)	1.19 (1.03-1.39))	1.07 (0.92-1.25)
	30-39/20-29	0.99 (0.96-1.01)	0.95 (0.92-0.98)	0.98 (0.95-1.00)	1.07 (1.04-1.10)
	40-49/20-29	1.34 (1.25-1.43)	1.12 (1.04-1.21)	1.26 (1.22-1.31)	1.29 (1.23-1.36)
	>=50/20-29			1.63 (1.48-1.80)	1.67 (1.50-1.85)
AD	<20/20-29	1.11 (0.96-1.27)	1.16 (1.00-1.35)	1.15 (0.88-1.51)	1.03 (0.79-1.36)
	30-39/20-29	1.11 (1.07-1.16)	0.99 (0.94-1.04)	1.13 (1.08-1.18)	1.15 (1.09-1.22)
	40-49/20-29	1.60 (1.43-1.78)	1.18 (1.05-1.33)	1.57 (1.47-1.67)	1.51 (1.39-1.64)
	>=50/20-29			2.18 (1.87-2.54)	2.01 (1.70-2.35)

*: Relative risks relative 20-29 year old; # adjusted for site-intercept, sex, birth year, age of the spouse; ASD: Autism Spectrum Disorder; AD: Autistic Disorder.

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5.5.1 Independent Effects of Paternal and Maternal age:

Figures 1 and **Figure 2** present the pattern of association between advancing paternal and advancing maternal age and risk for ASD adjusting for sex, birth year and the age of the spouse.Qualitatively, there was a statistically significant linear increase in the risk of ASD with increasing paternal age (**Figure 1**), and a statistically significant monotonic increase in the risk of ASD with increasing maternal age (**Figure 2**). This is quantified in risk estimates in **Table 2**. For example, relative to the group aged 20 to 29 years fathers older than 50 years had 1.7 fold (95% CI: 1.5-1.8) increased risk for having an offspring with ASD. Relative to the group aged 20 to 29 years mothers older than 40 years had 1.1 fold (95% CI: 1.1-1.2) increased risk of having an offspring with ASD.

There was also evidence for increased risk of ASD among younger mothers (**Figure 2**). Relative to the group aged 20 to 29 years mothers younger than 20 years had 1.3 times (95% CI: 1.2-1.4) increased risk of having an offspring with ASD (**Table 2**).

Similar risk pattern associations were evident for AD, but risk estimates were higher compared with ASD (**figure S1 and figure S2; table 2**)

Figure 1 Development of relative risk for ASD with increasing **paternal** age. Dotted lines indicate 2-sided 95% confidence intervals

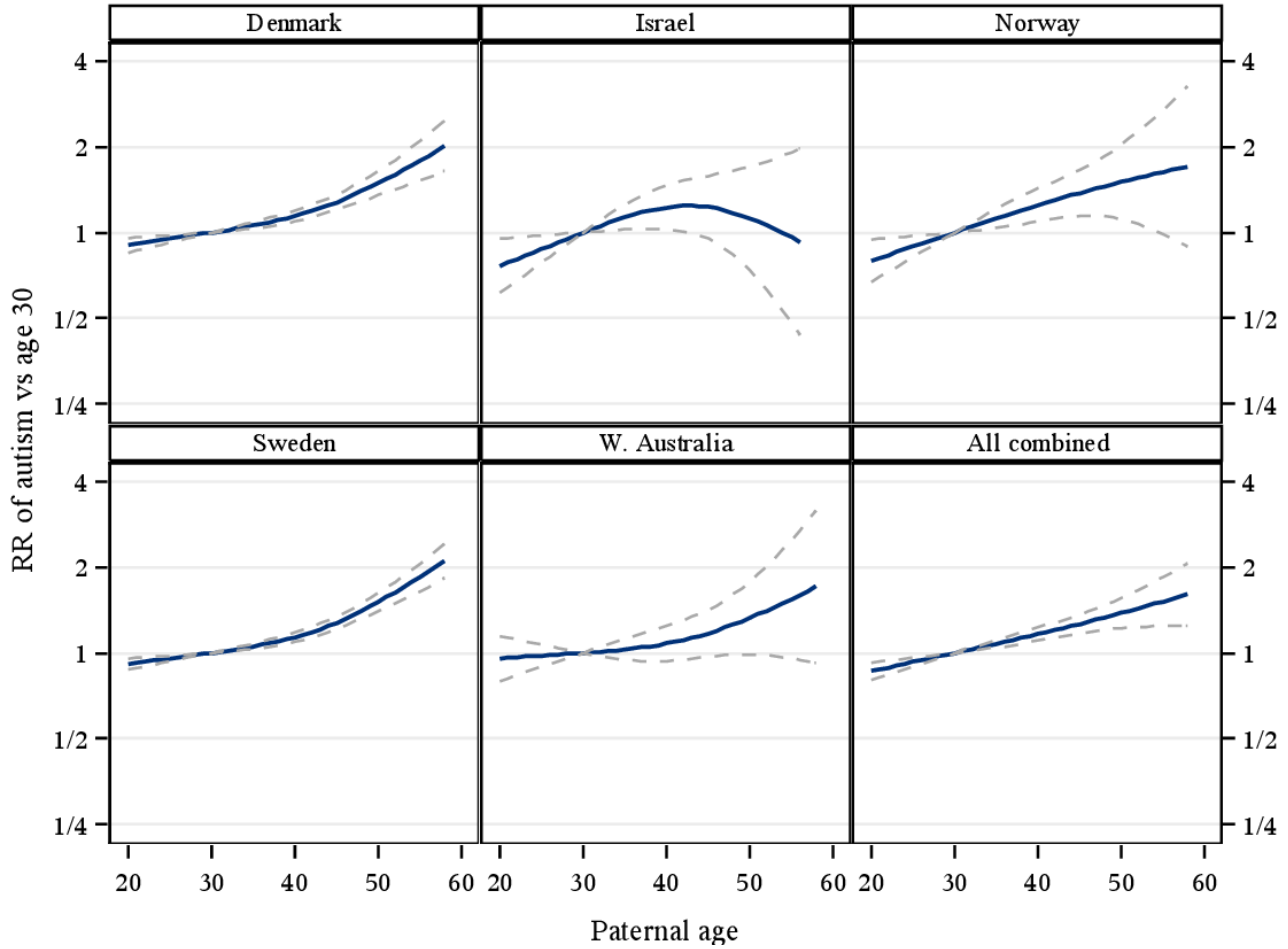


Figure 2 Development of relative risk for ASD with increasing **maternal** age. Dotted lines indicate 2-sided 95% confidence intervals

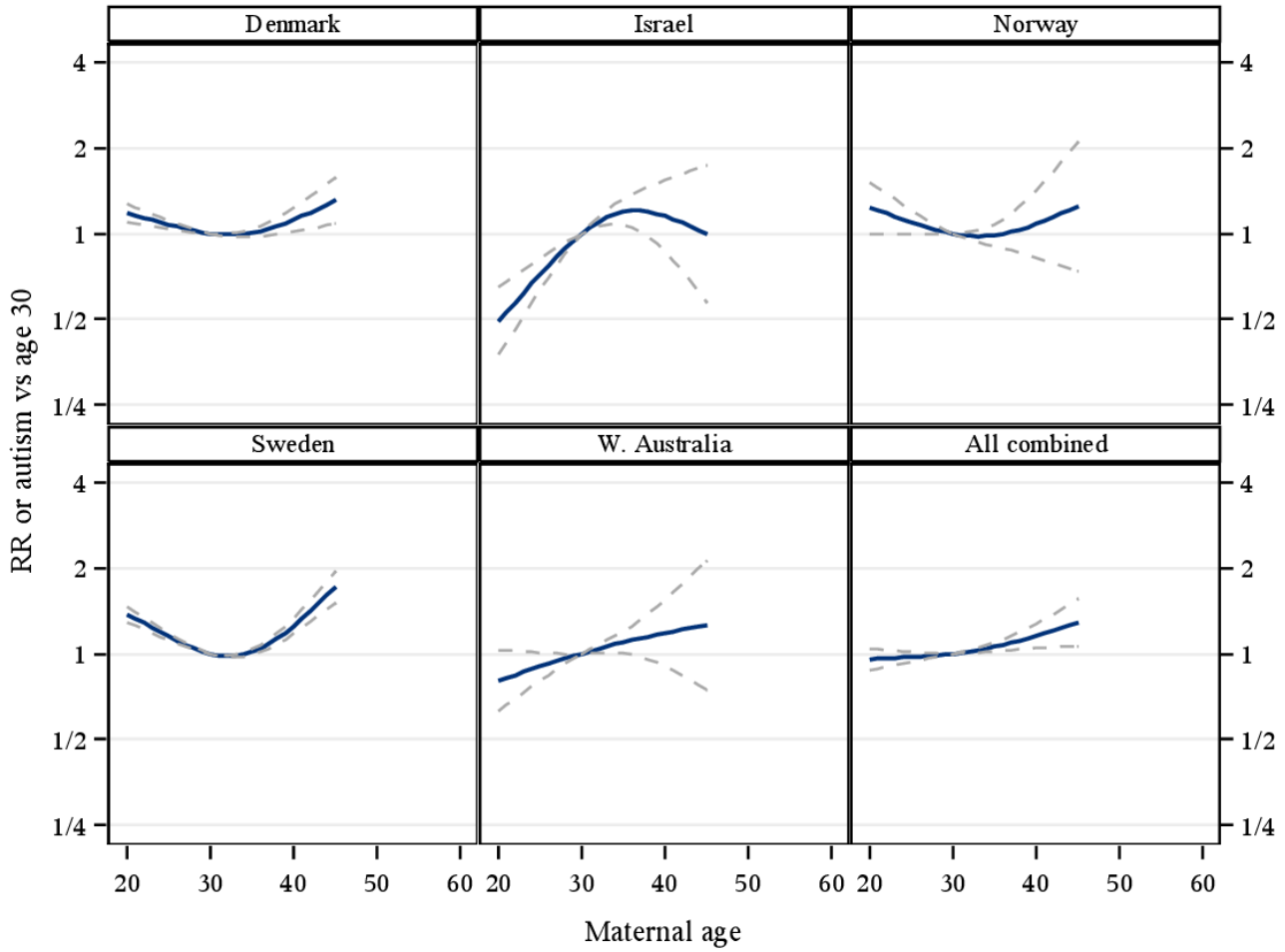


Figure S1 Development of relative risk for AD with increasing **paternal** age. Dotted lines indicate 2-sided 95% confidence intervals

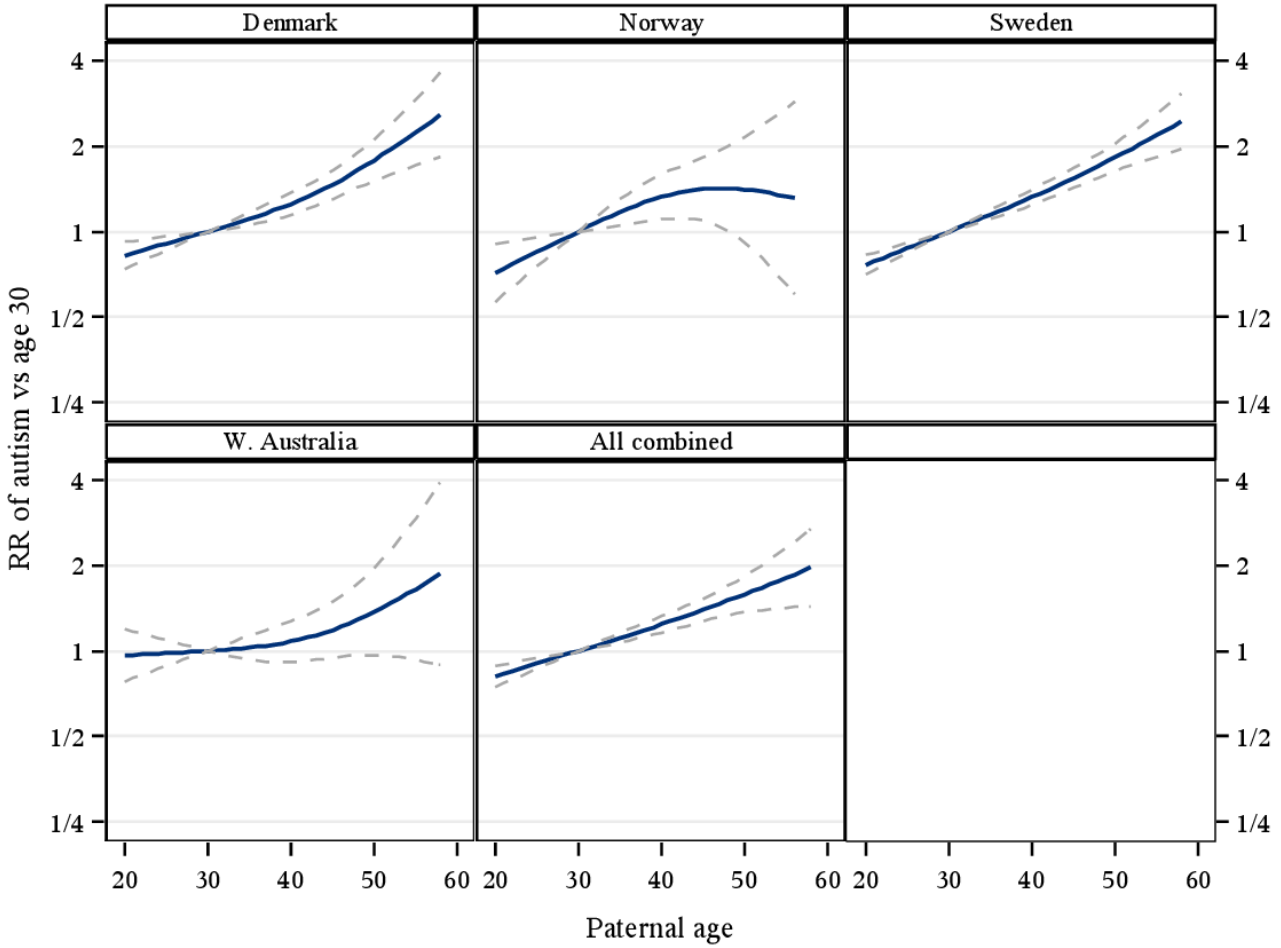
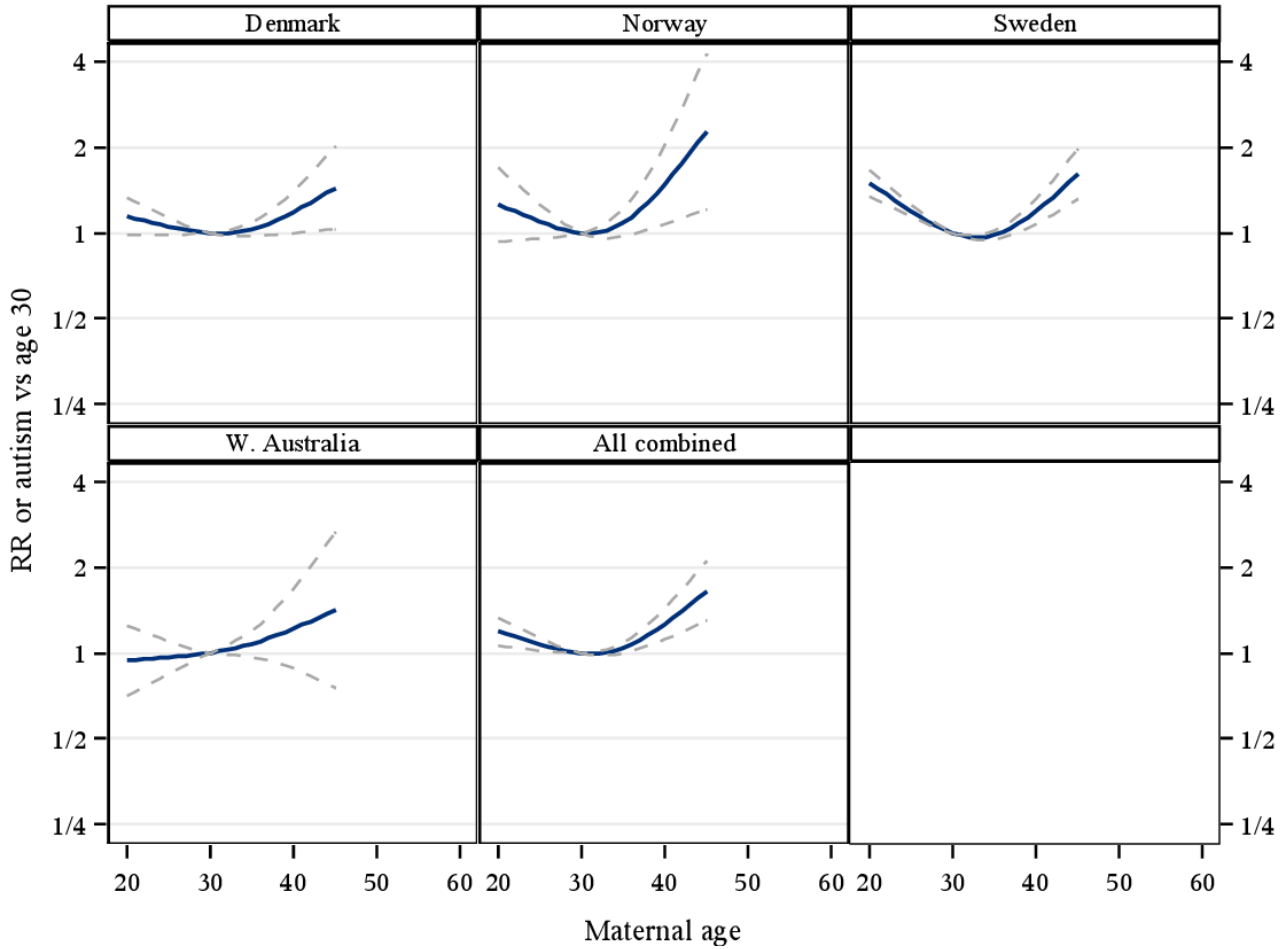


Figure S2 Development of relative risk for AD with increasing **maternal** age. Dotted lines indicate 2-sided 95% confidence intervals



5.5.2 Joint effects if Paternal and maternal age

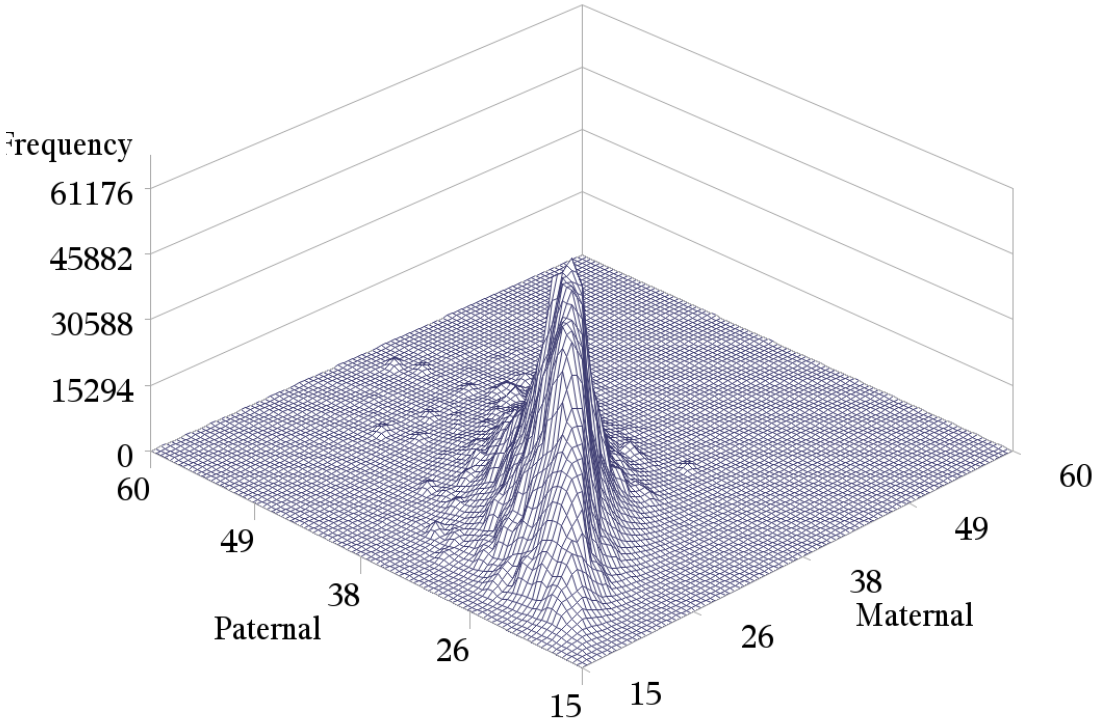
Couples consisting of mothers 20-40 years of age and fathers 20-50 of years of age generate 97% of all births. 2.2 of all births have a mother younger than 20 and 2.4% a mother older than 40. 0.9% of all births have a paternal age older than 50 (**table S1**).

Table S1 Number of births and percent of all births by categories of paternal and maternal age

Maternal age	Paternal age	Births	% of all birth
<20	<20	17555	0.304
	20-29	101783	1.765
	30-39	8019	0.139
	40-49	583	0.01
	>=50	82	0.001
20-29	<20	9535	0.165
	20-29	1838145	31.875
	30-39	1200406	20.816
	40-49	70466	1.222
	>=50	7311	0.127
30-39	<20	203	0.004
	20-29	214377	3.717
	30-39	1750204	30.35
	40-49	379095	6.574
	>=50	30745	0.533
>=40	<20	10	0
	20-29	2313	0.04
	30-39	34479	0.598
	40-49	90484	1.569
	>=50	10998	0.191

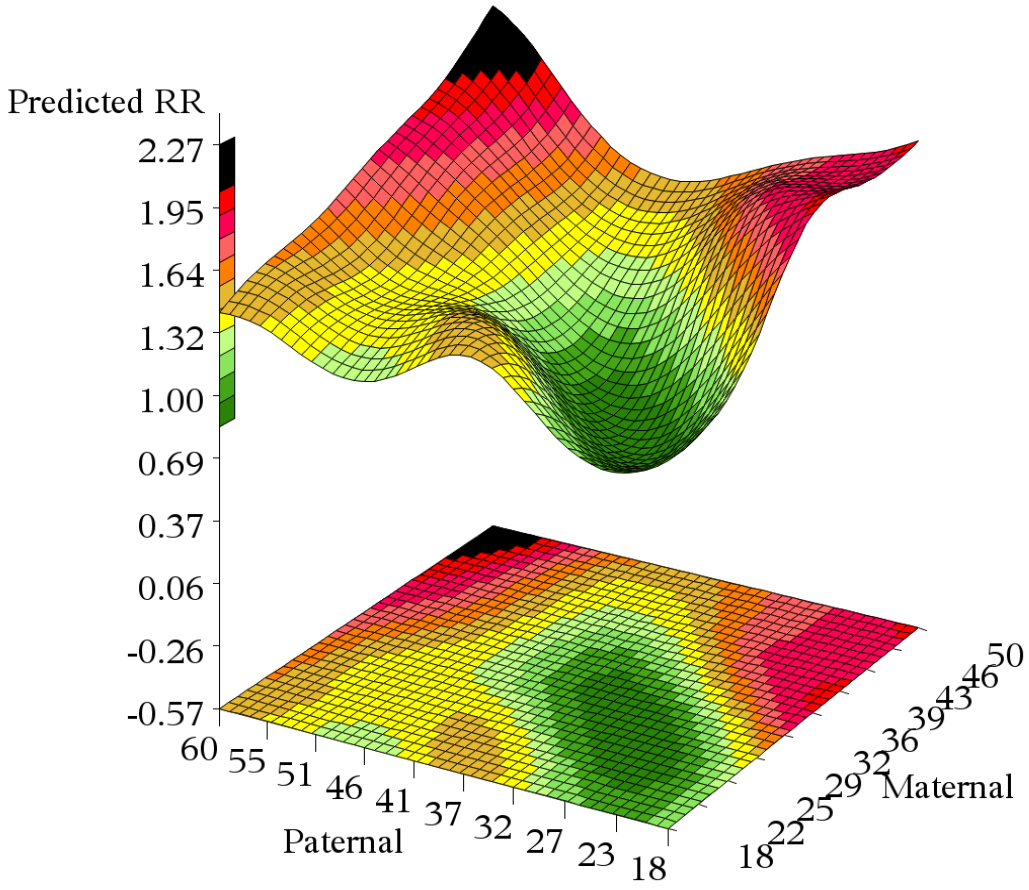
The bivariate distribution of number of births by paternal and maternal age across all sites is illustrated in **figure S3**.

Figure S3 Parental age distributions. Number of births by paternal and maternal age



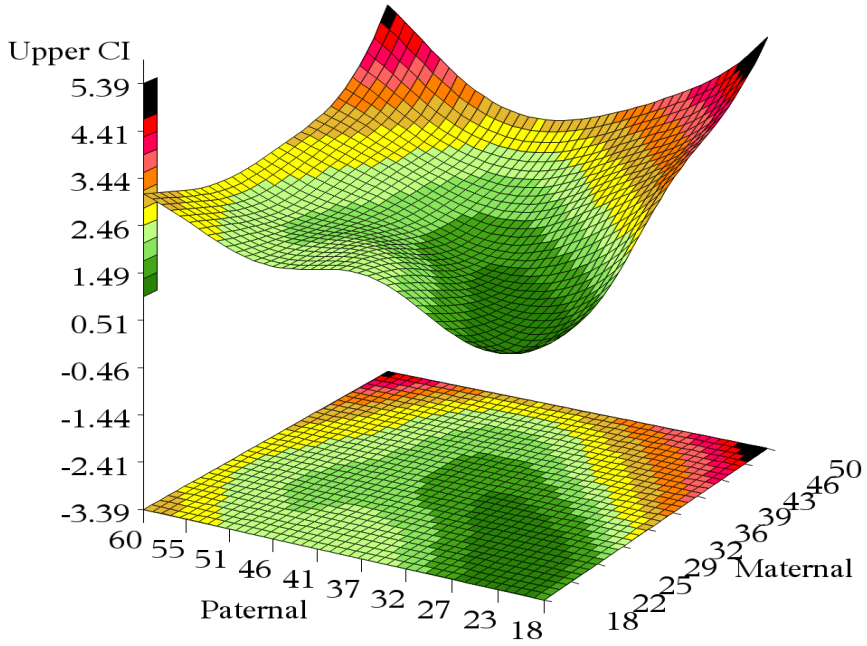
The ASD risk as a function of the joint exposure of paternal and maternal age (bivariate) is displayed in F**igure 3**. The figure present a 3D surface of the RR estimates for the different paternal-maternal age combinations. As reference group across the entire surface we used the couples where the father and mother were both equal to 25. Colours from light and very light green towards the yellow to red colour spectra indicate increased risk while dark green colour indicate decreased or equal risk compared with the couples of 25 year old parents.

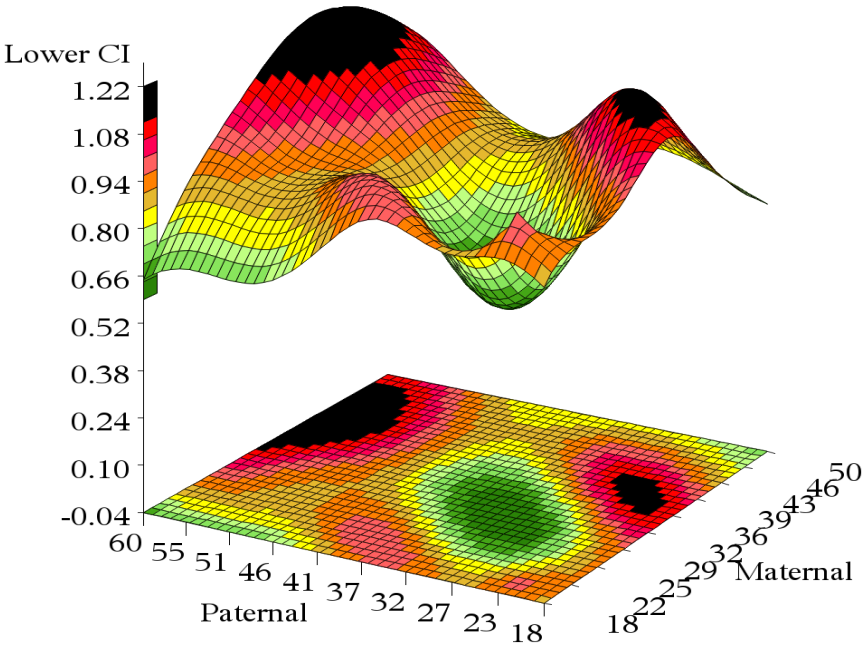
Figure 3 Relative risk (RR) of ASD by paternal and maternal age jointly



The surface planes generated by the corresponding point-wise 95% upper and lower confidence limits are presented in the **figure S7**.

Figure S7 Relative risk of ASD by paternal and maternal age jointly - Lower (lower panel) and upper (upper panel) 95% confidence limits



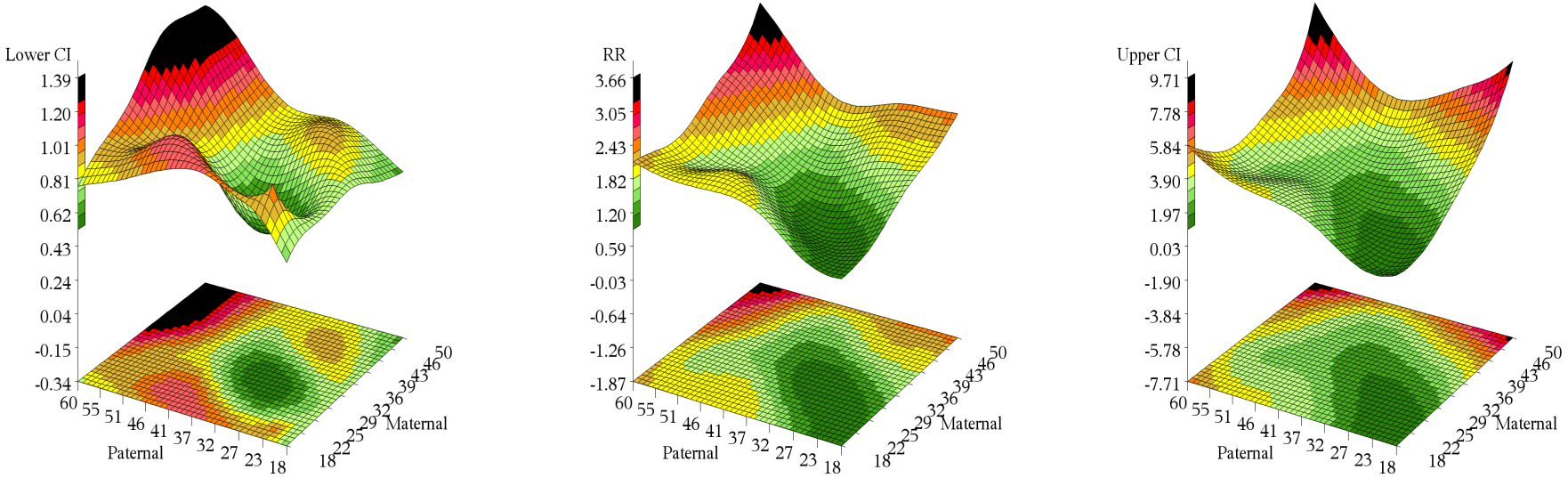


The paternal-maternal age associated risk of ASD in **Figure 3** show an inverse-shaped form compared to the age of mating distribution (**Figure S3**). Lowest ASD risk is concentrated in a central elliptic region corresponding to the ages of couples generating a majority of births, that is 29-39 year old fathers and 25-35 year old mothers. Risk of ASD increases in all directions from this region, that is with increasing age differences between the spouses.

Highest risk for ASD is seen in three regions approximated by (a) Fathers older than 45 independent of maternal age; (b) Fathers 35-45 with mothers <25; and (c) Mothers 25-40 years old with father ≥5 years younger. These three regions correspond to 3.0%, 3.4% and 0.8% of all birth.

The association between maternal and paternal age jointly and AD was similar to that of ASD (**figure S8**).

Figure S8 Relative risk of AD by paternal and maternal age jointly - Lower 95% confidence limit (left), RR point estimate (middle) and upper 95% confidence limit (right)



When evaluating which of the two components explain most of the variations, for ASD the direct ageing was more pronounced while for AD the direct ageing and difference in age components were equally important. However, there was strong support that both were important, jointly (**table S3**).

Table S3 Model selection. Goodness of fit comparing logistic regression models with and without parameters for mean parental age (mean of maternal and paternal age), the difference in parental age (maternal-paternal age) or both.

Model and model parameters	ASD		AD	
	AIC	Diff[#]	AIC	Diff[#]
f0: site + birth year + sex	363,640	0	139,226	0
f1: site + birth year + sex + mean	363,516	124	139,104	122
f2: site + birth year + sex + diff	363,509	131	139,106	120
f3: site + birth year + sex + mean + diff	363,437	203	139,036	190

#: AIC differences versus model f0

For AD the risk patterns was similar as that of ASD but with higher RR on more extreme ages of fertility.

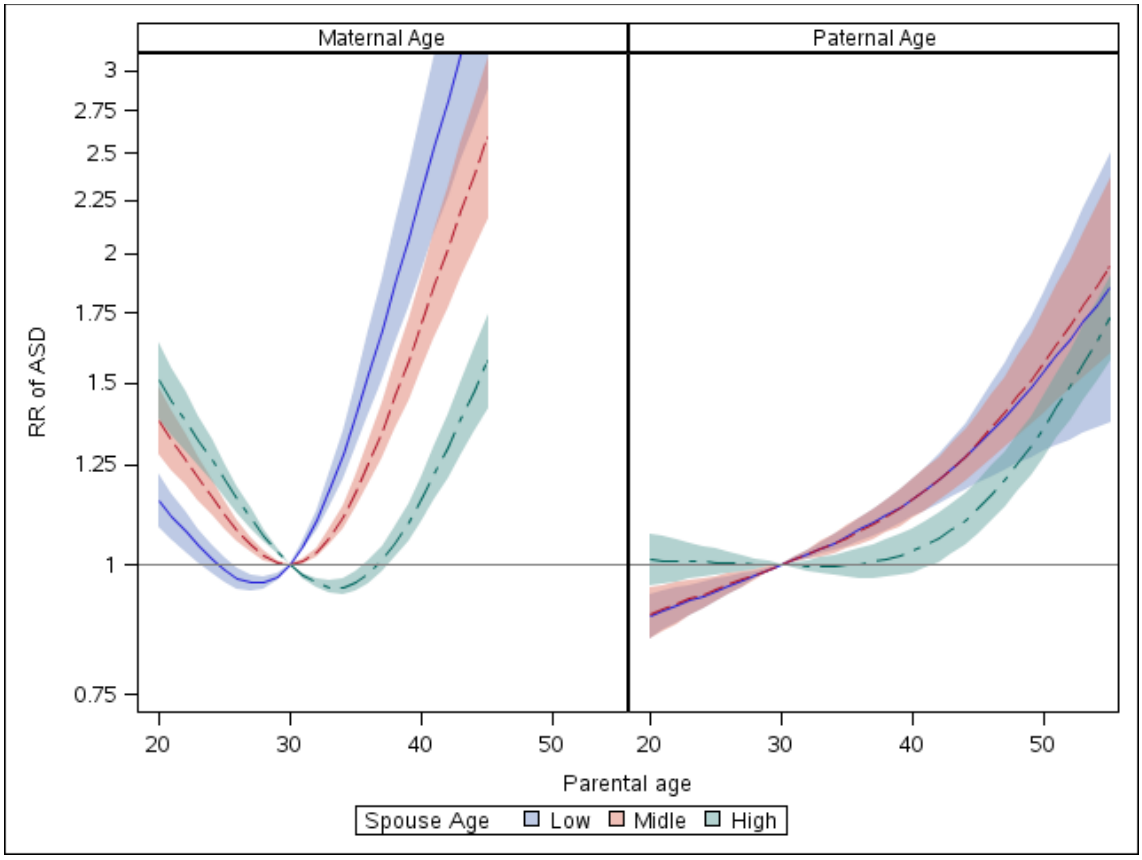
5.5.3 Subgroup analyses

The risk of ASD by advancing paternal age was evident in all three sub-group of maternal age (<27, 27-30 and >31) (**figure S5**). For couples where the mother was ≤ 31 there was a statistically significantly decreased risk for fathers less than 30 compared with fathers at age 30. For the sub-group of oldest mothers, >31, the effect of advancing paternal age was delayed compared with the sub-groups of mothers ≤ 31 and not visible before the father was 40.

Similar, for RR associations with maternal age in sub-groups of the father: #The risk of ASD by advancing maternal age was evident and similarly shaped in each sub-group of paternal age and <29, 29-34 and >34 (figure S5). However, as seen earlier in figure 3,

there is a pattern of higher risk for couples with bigger age difference between the father and the mother.

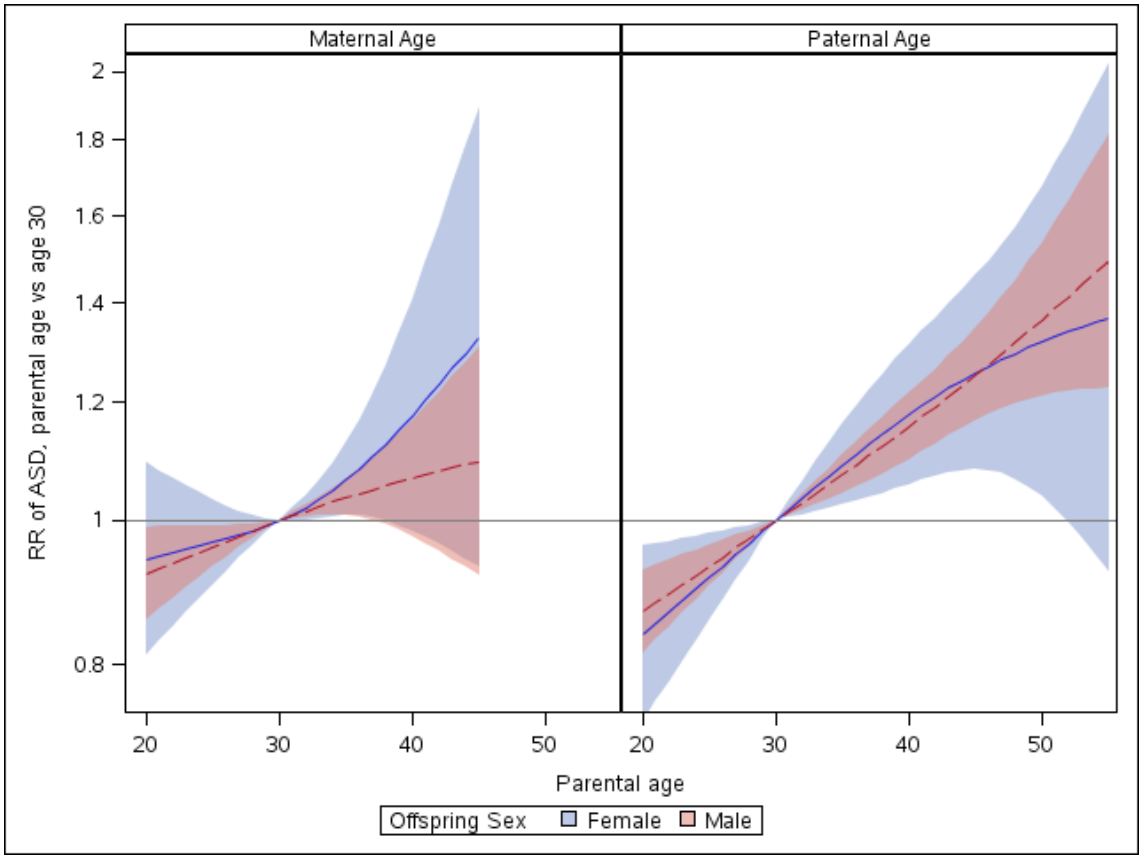
Figure S5 RR of ASD in subgroups of the parental age. Colour bands for 95% confidence intervals. Each sub-group contain one third of the cases.



5.5.4 Supplementary analyses

The relative risk were similar for male and female offspring (**figure S4**). Fitting Cox regression models to the data from Denmark and Sweden produced RR similar to the RR from the logistic regression.

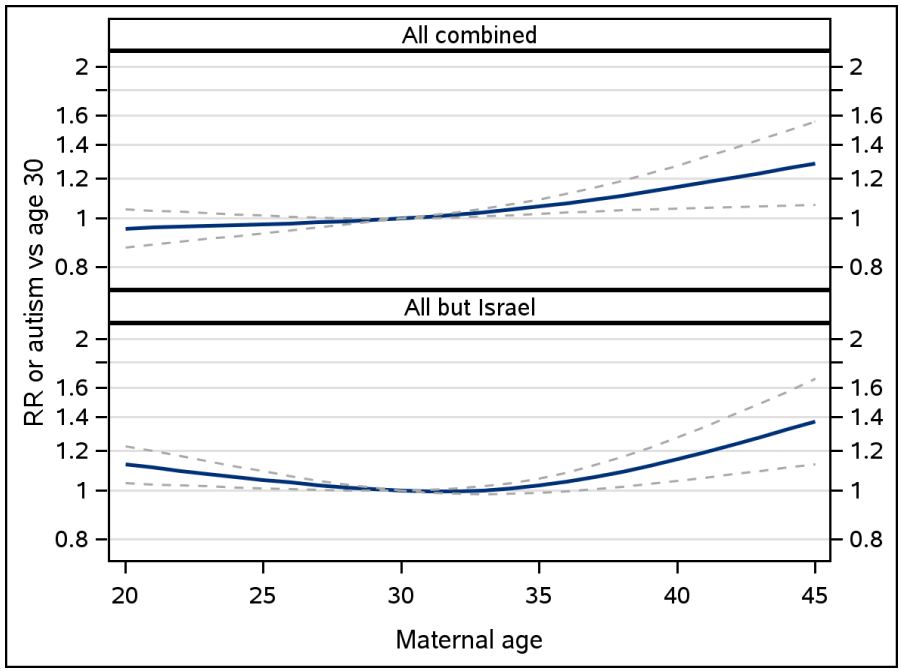
Figure S4 RR of ASD in by parental age for male (red) and female (blue) offspring separately. Colour bands for 95% confidence intervals.



Note: All RR are calculated relative age 30 within **each** sex-subgpoup **separately**, males separately from females.

The functional form of maternal age versus RR was similar across sites, only Israel showed any diverging shape for the maternal age by RR of ASD association. Combining all sites except Israel: The functional form of the maternal age by ASD relative risk development looked similar to when including Israel (**Figure S6**) and the 3D figure of the joint effect of paternal and maternal age on the risk of ASD was qualitatively similar (**Figure S9**).

Figure S6 Maternal age versus RR ASD combining all sites - With and without including Israel



5.6 Discussion

The results of this study provide the strongest evidence to-date supporting the hypothesis that older age of fathers, and older age of mothers at the time of birth of the offspring are both associated with risk for ASD in the offspring. Therefore representing independent risk factors. From the approximately age of 30, risk for ASD increased with increasing paternal or maternal age all the way up to the limits of male and female reproduction age. The magnitude of observed associations between parental age and risk for ASD were similar to those reported in earlier meta analyses (Sandin et al. 2012; Hultman et al. 2011), and were more pronounced for AD compared with ASD. Although it may seem that the RR associated with paternal age is higher than the RR for maternal age, this is due to the difference in child-bearing potential. When comparing fathers and mothers on the same age scale, from the age 30 to the age 45, the risk increase with advancing age was similar.

Results also showed evidence for an interaction effect of paternal and maternal age: The bivariate model showed lowest ASD risk in couples of similar age, risk increased when one spouse was a several years older than the other and was most pronounced when both parents where old, compared to having only one older parent in the

parents couple. There was no support for difference in associations for male or female offspring. The relative risk of ASD with parental age was similar across sites, using clinical registers as well as (public) insurance based registers, with some quantitative heterogeneity but not qualitatively.

The main mechanism proposed for the paternal age effect is through genomic alterations. This hypothesis postulates that de-novo mutations are responsible for the association between paternal age and ASD with increasing frequencies of mutations accumulating with advancing paternal age (Kong et al. 2012; Reichenberg et al. 2006). Recent human (Kong et al. 2012) and animal studies (Flatscher-Bader et al. 2011) provide support for this hypothesis. In contrast, mechanisms mediating the effect of advancing maternal age on risk for ASD have not been frequently investigated. Maternal age has been associated with chromosomal changes (Martin 2008; Ginsburg et al. 2000) and genomic modifications (Kaytor et al. 1997).

The study further provides new insights into potential aetiological factors in autism. Results showed that offspring of couples of moderately old fathers (aged 35-45) with substantially younger mothers (>10 years younger) as well as offspring of moderately old mothers (aged 30-40) with substantially younger fathers (>10 years younger) had increased risk for ASD. These results suggest that ASD aetiology may include the effects if social factors. Such factors, for example, traits related to the autism phenotype, may be present independently in fathers or mothers leading to delayed parenting (Croen et al. 2002) . Traits such as shyness and aloofness which may limit interactions with women have been described in fathers of autistic children (Eaton et al. 2001; Fombonne 2005). Alternatively, social factors may act jointly in the father and mother, representing assortative mating where individuals with similar traits (phenotype) and associated genotype mate, or secondary assortative mating between individuals with complementary traits (Merikangas 1982). Assortative mating has been associated with adverse outcome in the offpsring in both human and animal studies. In a large study from Britain increasing husband-wife height difference was associated with abnormal pregnancy outcomes (Mascie-Taylor & Boldsen 1988). Female and male mice that mated with preferred partners had higher reproductive success and better progeny performance than individuals mated with non-preferred partners (Drickamer

et al. 2003). Evidence for assortative mating has been documented in disorders with phenotypic similarity to ASD (Mataix-Cols et al. 2013). This intriguing possibility should be studied further because it has implications to both genetic studies and early detection and intervention.

Study results also suggest that younger age of parents may be associated with increased risk of ASD. Although not observed in meta-analysis of maternal age (Sandin et al. 2012), a study of two twin samples from Sweden and the UK showed an association between younger paternal age and risk of ASD (Lundström et al. 2010). While most focus has been on the effect of advancing parental age and risk to the offspring, our observation that young parental age, in particular maternal age, also increases risk for ASD, agree with epidemiological observations in several other disorders/traits. For example, a report a U-shaped risk profile for pre-term birth and birth weight in relation to paternal age (Abel et al. 2002). These observations are interesting given the suggested links between pre- and perinatal complications and the risk of developing ASD (Kolevzon et al. 2007).

From a mathematical statistical perspective a bias is defined as a numerical difference between the underlying true value of an estimator (such as the relative risk) and its estimate. Thus, it is a systematic error in the estimation of an effect. While randomization of treatments (exposures) to study participants offer a tool, while not a complete solution, to address bias problems it is not feasible in observational the epidemiological studies presented in this thesis.

The two major sources of bias in observational studies include selection bias and measurement errors.

Selection bias occur when the selection of the study population is not representative for the target population. Selection bias can occur in any study but is of special concern in case-control studies since selection is done conditional on the already observed outcome. In cohort studies bias could be introduced as a result of self-referral of participants but in a cohort study including the full population such as ours there are other possible sources.

One common source is non-response where exposure or outcome data are missing for

parts of the data. Another source of bias in the selection is the detection bias where exposed (or unexposed) subjects are followed or measured more intensively then the unexposed. With the registers collecting data in a publicly financed and utilized health system and with no private psychiatric health services the first source must be considered very unlikely since this cover the full population and the basic data collected is essentially complete. To what extent our results are biased by detection bias is harder to judge. It could for instance be argued that 'older parents' are more educated and experienced and as such better in getting access to health care and subsequent psychiatric diagnosis in their offspring than are the 'younger parents'.

As for selection bias measurement errors of both outcome and/or exposure variables can lead to biases in different directions. While our approach using full national cohorts with prospective follow-up using national registers avoids several possible errors such as recall bias due to self-report of data or due to biases introduced by interviewers, there are other sources that could have had severe consequences on our results. While the determination of parental age is exact using registers the (mis-) classification of autism can potentially be problematic. If the measurement error is independent of the exposure (“non-differential' errors) the effect is always a diluting effect of the effect size for dichotomous exposures and for most cases on non-dichotomous exposure. If the measurement error is different for different levels of exposure, e.g. more accurately diagnosed autism among older mothers than among younger mothers, the bias can be in any direction. To address the possible problems with the classification of children with autism we have analyzed autistic disorder (as well). Autistic disorder is the most severe form (sub-type) in the autism spectrum and as such can be expected to be more accurately classified.

A yet different type of bias in the study can be due to confounding where a variable is effecting both the exposure and the outcome. In a prospective cohort with essentially complete follow-up confounding is typically a variable occurring 'naturally'. In contrast to the other types of bias there are ways to deal with confounding in the analysis. For instance, in it's simplest form, if a confounding variable is present the effect of confounding can be addressed by including the covariate as a main effect in the model. Such an approach could also start with calculating an effect estimator in different sub-

categories of the confounding variable to ensure there is no interaction between the exposure and the confounding variable. Obvious confounders here is the birth year and calendar time, since both parental age and the rate of autism have increased dramatically over the last 20 years, and similarly did the age of partners. We adjusted for both these variables. It has been suggested to adjust for socio-economic status but we did not have that variable information available. Also, as I discuss above, it is not entirely clear if this should be done or not. Other suggestions have been to adjust for variables such as gestational age, birth weight or perinatal complications. However, all these variables can be considered to be on the causal path from exposure (parental age) to autism in the offspring and should therefore be approached with care. We decided not to include any such variable.

The epidemiological approach to dealing with the biases, including confounding, mentioned above is diverse and include among others (a) selecting the appropriate study population, (b) careful selection and execution (ideally prospective) of measurements, (b) try to achieve complete follow-up, (c) be careful not to design studies with high and uncontrolled drop-outs or missing values, (d) include important covariates (confounders or modifying effect) and (e) the choice of appropriate statistical methods.

To summarize, the study comes with several **strengths** such as the big sample size, prospective follow-up in national cohorts, consistent results across nations, geographic regions and different health and case-ascertainment systems while applying the unified and up-to-date statistical methods. Validation studies of the reported ASD diagnosis have been, or are being, performed in five sites with concordance of over 90% between the recorded and validation study diagnoses at the two sites that have concluded their studies (Schendel et al. 2013).

The study have some **limitations**. We lack several potentially confounding variables such as socio-economic status and parental psychiatric history. However, in earlier studies (Frans et al. 2013; Larsson et al. 2005) these confounders have not been shown to confound the relation between advancing paternal age and autism. In a recent Swedish study, partly overlapping with the Swedish data in the current study, the

increased risk among mothers < 20 was reduced in size and no longer statistically significant when adjusting for education and county of birth while the risk associated with advancing maternal age was increased (Frans et al. 2013). Availability of reliable and exact data for the date of diagnosis would have allowed a more detailed statistical adjustment for risk time and length of individual follow-up but the assumptions about the data distribution used here follow the main approach used by most earlier studies (Grether et al. 2009; Hultman et al. 2011; Reichenberg et al. 2006; Sasanfar et al. 2010) . Each parent will include on average 2-3 children but the lack of family data did not allow us to adjust for this in the analyses, again in agreement with practice in earlier studies.

5.7 Conclusion

Using population data from five nations and multiple geographic regions this study provides strong consistent evidence for a role for both advancing paternal and advancing maternal age in the etiology of autism. Advancing paternal age and advancing maternal age at the time of birth of offspring increases the risk of ASD. Younger maternal age may also be associated with increased ASD risk. De novo germline mutations, and toxic exposure may partly explain the observed associations. Since substantial differences in the ages of the parents are also associated with risk of ASD, this study suggests that social factors, in particular assortative mating may also play an important role

6 Study III - AD and ID after in-vitro-fertilization (IVF)

This section contain the manuscript published in The Journal of the American Medical Association under the title "Autism and Mental Retardation among Children Born After In-vitro Fertilization". It was Published July 3 2013.

Biography Sandin S, Nygren K-G, Iliadou A, Hultman CM, Reichenberg A. Autism and mental retardation among offspring born after in vitro fertilization. JAMA. 2013 Jul 3;310(1):75–84

DOI 10.1001/jama.2013.7222

The study was commented on in the JAMA editorial, doi:10.1001/jama.2013.7223

Note: This document differ only slightly from the submitted manuscript.

- The figures are in different form than the published. The figures in the published manuscript was edited by JAMA
- Single words can differ after the last edit by JAMA

6.1 Summary of the study

Importance

Between 1978 and 2010 approximately 5 million infants were born after in-vitro fertilization (IVF) treatments. Yet, there is limited information on neurodevelopment after IVF, especially after the first year of life.

Objective

To examine the association between use of any IVF and different IVF procedures and the risk of autistic disorder and mental retardation in the offspring.

Design, Setting and Participants

A population-based, prospective cohort study using Swedish national health registers. Children born between 1982 and 2007 were followed-up for a clinical diagnosis of autistic disorder or mental retardation until December 31st 2009. The exposure of interest was the IVF procedure, categorized according to whether intra cytoplasmic sperm injection (ICSI) (for male infertility) was used and whether embryos were fresh or frozen. For ICSI, whether sperm were ejaculated or surgically extracted sperm was also considered.

Main Outcome Measures(s)

Relative risks (RRs) for autistic disorder and mental retardation and rates per 100,000 person-years, comparing spontaneously conceived children with those born after an IVF procedure. Among those born after an IVF procedure, 5 procedures used in Sweden were compared with the most common treatment, IVF without ICSI with fresh embryo transfer. We also analyzed the sub-group restricted to singletons. We adjusted for sex, birth year, age of diagnosis, and parental age and psychiatric history.

Results

In Sweden, more than 2,5 million children were born between 1982 and 2007; 30,959 (1.2%) were born after an IVF procedure and followed for a mean of 10 (SD=6) years. Overall, there were 6,959 children with autistic disorder (103 [1.5%] born following IVF) and 15,830 with mental retardation (180 [1.2%] born following IVF). The RR for autistic disorder after any procedure compared with spontaneous conception was 1.14 ([95%

CI: 0.94-1.39], rates 19.0 vs 15.6 per 100,000 person-years) and for mental retardation, 1.18 ([95% CI:1.01-1.36, rates 46.3 vs 39.8). Overall, there were 6,737 singleton children with autistic disorder (54 [0.8%] born following IVF) and 15,279 with mental retardation (101 [0.7%] born following IVF). For both outcomes, there was no statistically significant association when restricting to singletons.

Compared with IVF without ICSI with fresh embryo transfer, there were statistically significantly increased risks for autistic disorder following ICSI using surgically extracted sperm and fresh embryos RR, 4.60 ([95% CI: 2.14-9.88], rates 135.7 vs 29.3 per 100,000 person-years); for mental retardation following ICSI using surgically extracted sperm and fresh embryos RR, 2.35 ([95% CI: 1.01-5.45], rates 144.1 vs 60.8); and following ICSI using ejaculated sperm and fresh embryos RR, 1.47 ([95% CI: 1.03-2.09], rates 90.6 vs 60.8). When restricting the analysis to singletons, the risks for autistic disorder associated with ICSI using surgically extracted sperm were not statistically significant, but the risks associated with ICSI using ejaculated sperm were significant for mental retardation (with frozen embryos, RR, 2.36 ([95% CI: 1.04-5.36], rates 118.4 vs 50.6); with fresh embryos, RR, 1.60 ([95% CI: 1.00-2.57], rates 80.0 vs 50.6)).

Conclusion and Relevance

In Sweden, compared with spontaneous conception, any IVF treatment was not associated with autistic disorder but was associated with a small but statistically significantly increased risk of mental retardation. Regarding specific procedures, the use of IVF with ICSI for paternal infertility was associated with a small increase in the relative risk for autistic disorder and mental retardation compared with IVF without ICSI. The prevalence of these disorders was low, and the increase in absolute risk associated with IVF was small. These associations should be assessed in other populations.

6.2 Introduction

Between 1978 and 2012, approximately 5 million infants worldwide were born following in-vitro fertilization (IVF). The original IVF procedure, allowing an egg to be fertilized by sperm in-vitro, is usually used in the absence of male-factor infertility. This procedure is used in Sweden in about half of all treatments. Embryos can be transferred immediately after fertilization (fresh) or frozen for later use. The introduction of the intra cytoplasmic sperm injection (ICSI) in 1992 (Palermo et al. 1992) , where a sperm is injected into an egg, allows treatment for male-factor infertility. For ICSI, sperm can be collected by ejaculation or surgical extraction.

Studies have demonstrated that IVF with or without ICSI is generally safe (Joint RCOG Document 2012) but can be associated with an increased risk for perinatal complications, including pre-term birth (Sazonova et al. 2011). Concern has been raised about ICSI in particular,(Anon 2004) which bypasses the natural selection of sperm, may physically damage the egg, and may contaminate the cytoplasm of the egg cell with culture media when the sperm is inserted. IVF procedures have also been associated with several neurological disorders, including cerebral palsy (Strömberg et al. 2002), Russell-Silver (Eroglu & Layman 2012), Beckwith-Wiedemann and Angelman syndromes (Eroglu & Layman 2012; Allen & Reardon 2005). No study has investigated the association between different IVF procedures and neurodevelopment, and few studies have investigated whether IVF treatments are associated with neurodevelopment after the first year of life (Hvidtjørn et al. 2009). Few studies have looked at autistic disorder and mental retardation, 2 of the most severe chronic developmental disorders, affecting 1% to 3% of all children in developed countries (Mash & Barkley 2002 pp362–389).

This prospective cohort study was designed to analyze the hypotheses that the use of any IVF procedure as well as specific procedures are associated with increased risk of autistic disorder and mental retardation in the offspring.

6.3 Methods

6.3.1 Study Population

A birth-cohort of all children born alive in Sweden from January 1, 1982 to December

31, 2007 was established using data from Swedish national registers, including the Medical Birth Register(Axelsson 2003), Multi-generation Register (Ekbom 2011) Patient Register (Ludvigsson et al. 2011; Sellgren et al. 2011; Ekholm et al. 2005) and the IVF Register (Appendix B, eTable 1). Children were followed up until December 31, 2009. The study was approved by the Swedish National Board of Health and Welfare, and the ethics committee at the Karolinska Institutet (Stockholm, Sweden).

6.3.2 Exposure

Information about IVF treatments (Appendix B, eTable 1) was obtained from the National Board of Health and Welfare. IVF without ICSI is used almost exclusively to treat female infertility, while IVF with ICSI is used for male infertility.

We classified mode of conception as spontaneous or IVF. IVF was further classified as using ICSI; and if ICSI was used, by the source of sperm, ejaculated or surgically extracted. Treatment with surgically extracted sperm was introduced 1996. Embryos can either be cultured in-vitro for 2-3 days (cleavage-stage) or for 5-6 days (blastocyst). During treatment, several embryos are often produced. The embryos not immediately used can be frozen. IVF procedures were also classified by whether the embryo was fresh or frozen. Thus, 6 procedures currently used in Sweden were considered: (1) IVF without ICSI with fresh embryo transfer; (2) IVF without ICSI with frozen embryo transfer; (3) ICSI using ejaculated sperm with fresh embryos; (4) ICSI with ejaculated sperm and frozen embryos; (5) ICSI with surgically extracted sperm and fresh embryos, and (6) ICSI with surgically extracted sperm and frozen embryos.

6.3.3 Outcome

Autistic disorder is characterized by deficits in social interaction, communication, and restricted, stereotypical or repetitive behavior. Mental retardation is defined as an IQ below 70 and limitations in adaptive behavior. In Sweden, all infants and preschool children are regularly seen at well-child care clinics and undergo routine medical and developmental screening. At age 4 years, a mandatory developmental assessment (motor, language, cognitive and social development) is conducted. Children with a suspected developmental disorder are referred for further assessment by a specialized team. Diagnostic information is reported to the Patient Register.

The International Classification of Diseases, 9th and 10th revisions were used. We focused on mental retardation and on the narrow diagnosis of infantile/childhood autism (diagnostic codes ICD-9 299A or ICD-10 F84.0), and do not include other forms of autism spectrum disorders.

6.3.4 Covariates

We considered several factors that might confound or modify the association between IVF treatments and autistic disorder or mental retardation in the child. Parental psychiatric history (Larsson et al. 2005) was classified as present/not-present for each parent separately using any diagnosis at any time before the birth of the child (for ICD-codes see Appendix B, eTable 2). We also obtained information on parental age (Hultman et al. 2011; Grether et al. 2009; Sandin et al. 2012), birth year, multiple births, and preterm birth (Sazonova et al. 2011) (<week 37). Multiple births and preterm birth may lay on a causal pathway to adverse developmental outcome, and were therefore examined as effect modifiers.

6.3.5 Statistical methods

First we examined the association between any IVF procedure and autistic disorder and mental retardation compared with spontaneous conception. This is the most important comparison from a public health perspective. Second, as there may be different risks associated with different procedures or parental factors underlying the choice of procedure, we analyzed the association between 5 of the different IVF categories and autistic disorder and mental retardation compared with the most commonly used and least complicated procedure, IVF without ICSI with fresh embryo transfer. As couples undergoing fertility treatment may share common risk factors the relevant comparison group consists of other couples undergoing IVF treatment (Carson et al. 2010) which controls for reasons for infertility. We also provide the results using spontaneous conception as the reference.

We also combined data to investigate procedures with similar underlying parental factors and to increase power: all ICSI procedures, regardless of sperm source and type of embryo;, frozen embryos, regardless of type of IVF procedure; and surgical extraction of sperm, regardless of embryo type. In an exploratory analysis, we also

examined whether the embryo was transferred at the cleavage or blastocyst stage; blastocyst transfer data was available from 2002.

For descriptive purposes, we calculated the rate and the percentage of children with autistic disorder and mental retardation and exact 95% confidence intervals (CIs) (Clopper, C J & Pearson, E S 1934).

Using Poisson regression (SAS GLIMMIX version 9.3), we estimated RRs and 2-sided 95% Wald CIs. We fitted regression models by splitting the follow-up-time (child attained age between cohort entry and cohort exit) into 1-year intervals. Poisson regression gives approximately the same parameter estimates and likelihood ratios as Cox proportional hazards regression when the length of follow-up is split into finer intervals but allows for greater flexibility in the modeling (Whitehead 1980) . For each child and outcome, we only considered the first event. Each child was followed from age 1.5 years up to death, emigration from Sweden, onset of disease, the age of 28 years, or December 31, 2009, whichever came first. We first fitted crude models including covariates for exposure together with sex, birth year and attained age, then adjusted models including the potential confounding covariates parental age (paternal: <30, 30-39, 40-49 and ≥50; maternal: <25, 25-29, 30-34, ≥35) and paternal and maternal psychiatric history at offspring birth (yes/no). This set of models was fitted for the comparisons of any IVF procedure vs spontaneous conception as well as for the comparison of specific procedures.

To allow the most efficient adjustment of the time variables, attained age and birth year, we fitted natural cubic splines (Hastie et al. 2009; Benedetti & Abrahamowicz 2004) allowing for adjustment without assuming a specific functional form such as linear or stepwise.

All statistical tests were done on the 2-sided 5% level of significance. All RR are presented together with absolute rates per 100,000 person years adjusted for birth year, sex and age.

6.3.6 Supplementary analyses

To check for confounding potentially present in an observational study such as this and to allow a better understanding of the observed associations, we performed a-priori

specified analyses.

To confirm associations were not due to temporal trends, crude models were adjusted for calendar time using splines. To allow the treatment comparisons to be summarized with a single RR, we examined IVF procedure-by-age-interactions, allowing for different RRs at different ages. We calculated RR separately for male and female offspring.

At the first prenatal visit, mothers are asked about length of involuntary infertility. Additional models included this variable as a linear continuous confounder. Women are also asked about hormone treatment as the only fertility treatment; we compared children of these women vs spontaneous conception fitting crude and adjusted models.

We fitted a separate set of models also adjusting for certain genetic diseases and disorders (Appendix B, eTable 2) with known phenotypic and genetic overlap with autistic disorder and mental retardation (Hollander et al. 2010) .

Finally, we repeated all analyzes described above restricted to singletons.

For model checking purposes we fitted supplementary models (Fitzmaurice et al. 2004) assuming independence between families and a common correlation within and not requiring the data to follow a particular parametric distribution. We added several analyzes post hoc: To complement the analyzes of IVF procedures we calculated RR using spontaneous conceived children as control group. We analyzed the last 9 birth cohorts as a subgroup. To facilitate interpretation of the mechanism of the associations, we fitted the models separately to the subgroups of pre-term and term children. In yet separate analyzes we calculated and compared RR for multiple births after IVF and for multiple births after spontaneous conception.

6.4 Results

Characteristics of the children are presented in Table 1. A total of 2,541,125 children were alive at 1.5 years of age and had complete data on all the covariates; 30,959 (1.2%) were born following an IVF procedure. 18,288 children (0.7%) had missing information on parental age or term/pre-term status and were not included (65 with autistic disorder and 204 with mental retardation, including 1 and 2 cases among those born after an IVF procedure. After 1998 44% of infertility treatments have used ICSI for

male reproduction problems and frozen embryos increased from 9% before 1998 to 26% after 2005 (Table 1).

Autistic disorder was diagnosed in 6,959 children and mental retardation in 15,830; 103 (1.5%) and 180 (1.1%), respectively, were born after an IVF procedure. Cases had a mean follow-up time of 10 years (SD=6) , median 14 years (range, 0.1 to 26.5 years). The rates (per 100,000 person-years) of autistic disorder and mental retardation among spontaneous conceived children were 20.2 and 46.1, respectively. The highest rates of autistic disorder (215.0) and mental retardation (161.2) were in children born following ICSI using surgically extracted sperm with fresh embryos (Table 2).

Table 1 All children. Distribution of confounders and children characteristics for spontaneous conceived, across IVF procedures and for women with hormone treatment as the only fertility treatment.

Variable	Spontaneously Conceived	IVF without ICSI, Fresh embryo	IVF without ICSI, Frozen embryo	ICSI, Fresh embryo	ICSI, Frozen embryo	ICSI, Fresh embryo, surgically extracted sperms	ICSI, Frozen embryo, surgically extracted sperms
Number of Children (%boys)	2,510,166 (51.4)	16,668 (52.9)	2,777 (51.1)	9,241 (49.7)	1,477 (49.6)	628 (49.3)	168 (55.4)
Father Psych. History, N (%)	36,405 (1.5)	183 (1.1)	39 (1.4)	136 (1.5)	12 (0.8)	9 (1.4)	8 (4.8)
Mother Psych. History, N (%)	46,366 (1.8)	363 (2.2)	51 (1.8)	157 (1.7)	30 (2.0)	12 (1.9)	2 (1.2)
Pre-term, N (%)	143,688 (5.7)	3,631 (21.8)	435 (15.6)	1,562 (16.9)	190 (12.9)	107 (17.1)	17 (10.1)
Multiple Birth, N (%)	54,673 (2.18)	5,285 (31.7)	606 (21.8)	2,379 (25.7)	258 (17.5)	164 (26.1)	39 (23.2)
Birth year, Median (Min-Max)	1994 (1982-2007)	2000 (1982-2007)	2004 (1990-2007)	2002 (1992-2007)	2004 (1988-2007)	2002 (1996-2007)	2004 (1996-2007)

IVF: In Vitro Fertilization, ICSI: Intra Cytoplasmic Sperm Injection, N: Number of children; pre-term: before week 37

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Variable		Spontaneously Conceived	IVF without ICSI, Fresh embryo	IVF without ICSI, Frozen embryo	ICSI, Fresh embryo	ICSI, Frozen embryo	ICSI, Fresh embryo, surgically extracted sperms	ICSI, Frozen embryo, surgically extracted sperms
Maternal age distribution, N (%)	<25	532,141 (21.2)	128 (0.8)	19 (0.7)	183 (2.0)	18 (1.2)	14 (2.2)	3 (1.8)
	25-29	889,571 (35.4)	2,268 (13.6)	295 (10.6)	1,788 (19.3)	234 (15.8)	121 (19.3)	37 (22.0)
	30-34	734,554 (29.3)	7,254 (43.5)	1,113 (40.1)	3,970 (43.0)	637 (43.1)	280 (44.6)	73 (43.4)
	>34	353,900 (14.1)	7,018 (42.1)	1,350 (48.6)	3,300 (35.7)	588 (39.8)	213 (33.9)	55 (32.7)
Paternal age distribution, N (%)	<30	969,915 (38.6)	1,543 (9.3)	183 (6.6)	943 (10.2)	116 (7.8)	38 (6.0)	14 (8.3)
	30-39	1,290,362 (51.4)	11,660 (70.0)	1,889 (68.0)	6,181 (66.9)	979 (66.3)	365 (58.1)	96 (57.1)
	40-49	227,723 (9.1)	3,248 (19.5)	647 (23.3)	1,892 (20.5)	340 (23.0)	182 (29.0)	45 (26.8)
	≥50	22,166 (0.9)	217 (1.3)	58 (2.1)	225 (2.4)	42 (2.8)	43 (6.8)	13 (7.7)
Children born after IVF/ICSI (% in each birth year interval)	1983-1987		142 (100.0)	0	0	0	0	0
	1988-1992		1,588 (93.5)	105 (6.2)	4 (0.2)	2 (0.1)	0	0
	1993-1997		4,718 (68.5)	572 (8.3)	1,412 (20.5)	116 (1.7)	54 (0.8)	14 (0.2)
	1998-2002		4,577 (48.4)	502 (5.3)	3,665 (38.8)	388 (4.1)	286 (3.0)	39 (0.4)
	2003-2007		5,643 (44.2)	1,598 (12.5)	4,160 (32.6)	971 (7.6)	288 (2.3)	115 (0.9)
Years of involuntary infertility, Median (10th-90th percentiles)		0 (0-0)	3 (0-8)	2 (0-7)	3 (0-7)	2 (0-6)	2 (0-7)	2 (0-6)

IVF: In Vitro Fertilization, ICSI: Intra Cytoplasmic Sperm Injection, N: Number of children; pre-term: before week 37

Table 2 All children. Autistic Disorder and Mental Retardation. Number of cases, Person year of follow-up, Percent of children diagnosed with each disease, Rate (unadjusted) and Rate adjusting for age, sex and birth year.

	Autistic Disorder					Mental Retardation				
	Cases	**Person years**	**Percent (95% CI)[###]**	**Unadjusted[#] Rate per 100,000 person year (95% CI)**	**Adjusted[##] Rate per 100,000 person year (95% CI)**	**Cases**	**Person years**	**Percent (95% CI)[###]**	**Unadjusted[#] Rate per 100,000 person year (95% CI)**	**Adjusted[##] Rate per 100,000 person year (95% CI)**
Children born after IVF treatment and after Spontaneous Conception										
Spontaneous Conception	6,856	33,994,678	0.27 (0.27- 0.28)	20.2 (19.7- 20.7)	15.6 (15.1- 16.1)	15,650	33,947,960	0.62 (0.61- 0.64)	46.1 (45.4- 46.8)	39.8 (39.0- 40.5)
IVF	103	231,118	0.33 (0.27-0.40)	44.6 (36.7- 54.1)	19.0 (15.7- 23.2)	180	230,710	0.58 (0.50- 0.67)	78.0 (67.4- 90.3)	46.3 (40.0- 53.7)

NA: Not application since no cases, CI: Two-sided confidence interval, IVF: In Vitro Fertilization, ICSI: Intra Cytoplasmic Sperm Injection, Person Years: Year each live born child is contributing with in the analysis; Percent of children calculated as number of children observed with a disease diagnosis divided with the number of children born and reaching the age 1.5, #: Unadjusted rates, ##: Age, Birth year and sex adjusted rates; ###: Exact confidence intervals calculated on crude proportion autistic disorder and mental retardation without adjusting for possible confounding.

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	Autistic Disorder					Mental Retardation				
	Cases	**Person years**	**Percent (95% CI)[###]**	**Unadjusted[#] Rate per 100,000 person year (95% CI)**	**Adjusted[##] Rate per 100,000 person year (95% CI)**	**Cases**	**Person years**	**Percent (95% CI)[###]**	**Unadjusted[#] Rate per 100,000 person year (95% CI)**	**Adjusted[##] Rate per 100,000 person year (95% CI)**
Children born after specific IVF procedures										
IVF without ICSI, Fresh embryo	53	144,207	0.32 (0.24-0.42)	36.8 (28.0-48.2)	29.3 (21.2-40.4)	94	143,924	0.56 (0.46-0.69)	65.3 (53.3-80.0)	60.8 (49.1- 75.3)
IVF without ICSI, Frozen embryo	10	17,121	0.36 (0.17-0.66)	58.4 (31.3-109.0)	42.4 (22.1-80.9)	13	17,095	0.47 (0.25-0.80)	76.0 (44.0-131)	69.0 (36.9-119.5)
ICSI, fresh embryo	31	58,262	0.34 (0.23-0.48)	53.2 (37.3-75.8)	34.0 (22.1- 52.5)	59	58,177	0.64 (0.49-0.82)	101.4 (78.5-131)	90.6 (68.4-120.1)
ICSI, frozen embryo	1	7,022	0.07 (0-0.38)	14.2 (2.0-102.3)	9.4 (1.3-67.8)	8	7,005	0.54 (0.23-1.06)	114.2 (56.9-229)	103.9 (51.2-210.6)
ICSI, fresh embryo, surgically extracted sperm	8	3,720	1.27 (0.55-2.49)	215.0 (107.1-431.8)	135.7 (64.6-285.0)	6	3,722	0.96 (0.35-2.07)	161.2 (72.1-361)	144.1 (64.1-324.3)
ICSI frozen, embryo, surgically extracted sperm	0	787	0 (0-2.17)	NA	NA	0	787	0 (0-2.17)	NA	NA

Below only adjusted RRs are presented. Crude RRs are presented in Figures 1, 2 and 3. All results, including supplementary analyzes, are presented in the online tables (indicating supplementary results using italic font).

Compared with children born following spontaneous conception, those born after any IVF procedure had a statistically significantly increased risk of mental retardation RR, 1.18 ([95% CI: 1.01-1.36], rates 46.3 vs 39.8). The RR for autistic disorder was 1.14 ([95% CI: 0.94-1.39], rates 19.0 vs 15.6). For both mental retardation and autistic disorder, the risk estimates were slightly lower in singletons and not statistically significant RR, 1.01 ([95% CI: 0.83-1.24], rates 38.8 vs 38.5) and RR, 0.89 ([95% CI: 0.68-1.17], rates 14.4 vs 15.0) respectively (Appendix B, eTable 3 and eTable 4).

There was a statistically significantly increased risk for autistic disorder after ICSI using surgically extracted sperm with fresh embryos RR, 4.60 ([95% CI: 2.14-9.88], rates 135.7 vs 29.3) compared with children born after IVF without ICSI with fresh embryos. In children born preterm, the RR was 9.54 ([95% CI: 3.43-26.57], rates 364.5 vs 38.4). The increase in risk was not evident in singletons RR, 0.95 [95% CI: 0.13- 7.09], rates 21.9 vs 23.9) (Appendix B, eTable 5).

There was increased risk of mental retardation in children born after ICSI using surgically extracted sperm with fresh embryos compared with children born after IVF without ICSI with fresh embryos RR, 2.35 ([95% CI:1.01- 5.45], rates 144.1 vs 60.8). In children born preterm, the RR was 4.38 ([95% CI:1.53-12.48], rates 413.9 vs 92.2). The increase in risk was not evident in singletons RR, 0.70 ([95% CI: 0.10- 5.16], rates 36.1 vs 50.6).

There was increased risk of mental retardation in children born after ICSI using ejaculated sperm with fresh embryos RR, 1.47 ([95% CI: 1.03- 2.09], rates 90.6 vs 60.8) but not frozen. This risk increase was present also in singletons RR, 1.60 ([95% CI: 1.00-2.57], rates 80.0 vs 50.6). Risk for mental retardation was also statistically significant in singletons after ICSI using ejaculated sperm with frozen embryos RR, 2.36 [95% CI: 1.04-5.36], rates 118.4 vs 50.6)) but not among all children. For other procedures, the RR was not statistically significant (Appendix B, eTable 6).

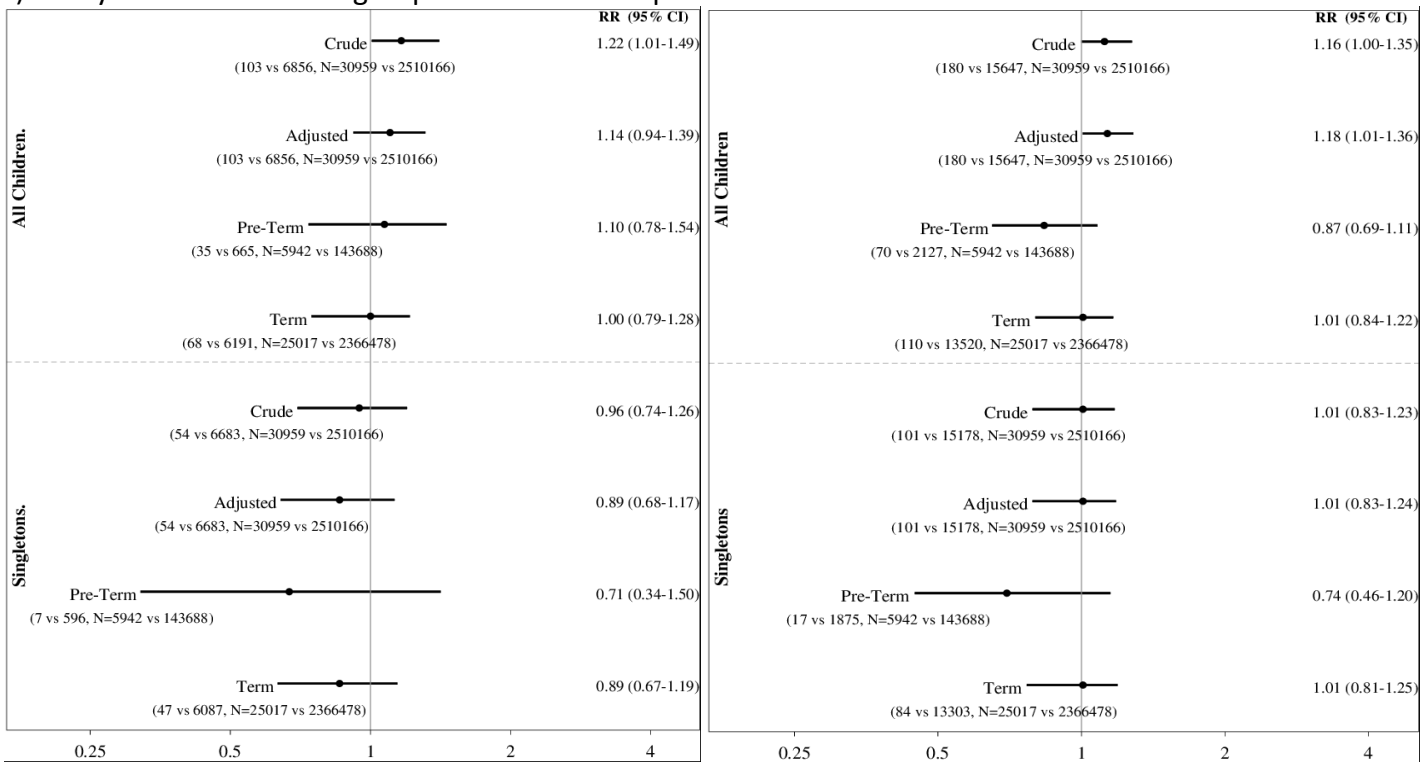
To further elucidate the effect of specific techniques, we analyzed combined

procedures. For autistic disorder, comparing the 2 procedures involving surgically extracted sperm with the 4 procedures involving ejaculated sperm, there was an increase in risk associated with the surgical extraction RR, 3.29 ([95% CI:1.58- 6.87], rates 110.1 vs 30.9). The risk was even higher among preterm births RR, 8.06 ([95% CI: 2.97-21.85], rates 319.8 vs 42.3) but was reduced in magnitude and was no longer statistically significant when restricted to singletons RR, 0.73 ([95% CI: 0.10- 5.30], rates 18.3 vs 24.3) (Appendix B, eTable 7).

For mental retardation, comparing the 4 different ICSI procedures with the 2 procedures without ICSI, there was an increased risk RR, 1.51 ([95% CI: 1.10- 2.09], rates 93.5 vs 61.8). The risk increase was similar in singletons RR, 1.50 ([95 CI: 0.98-2.29], rates 80.2 vs 54.8) and among preterm births RR, 1.73 ([95% CI: 1.05-2.86], rates 166.7 vs 96.0). The risk increase comparing procedures using surgical extraction vs ejaculated sperm was present only for mental retardation among preterm births RR, 3.31 ([95% CI: 1.18-9.31], rates 356.7 vs 109.4) (Appendix B, eTable 8).

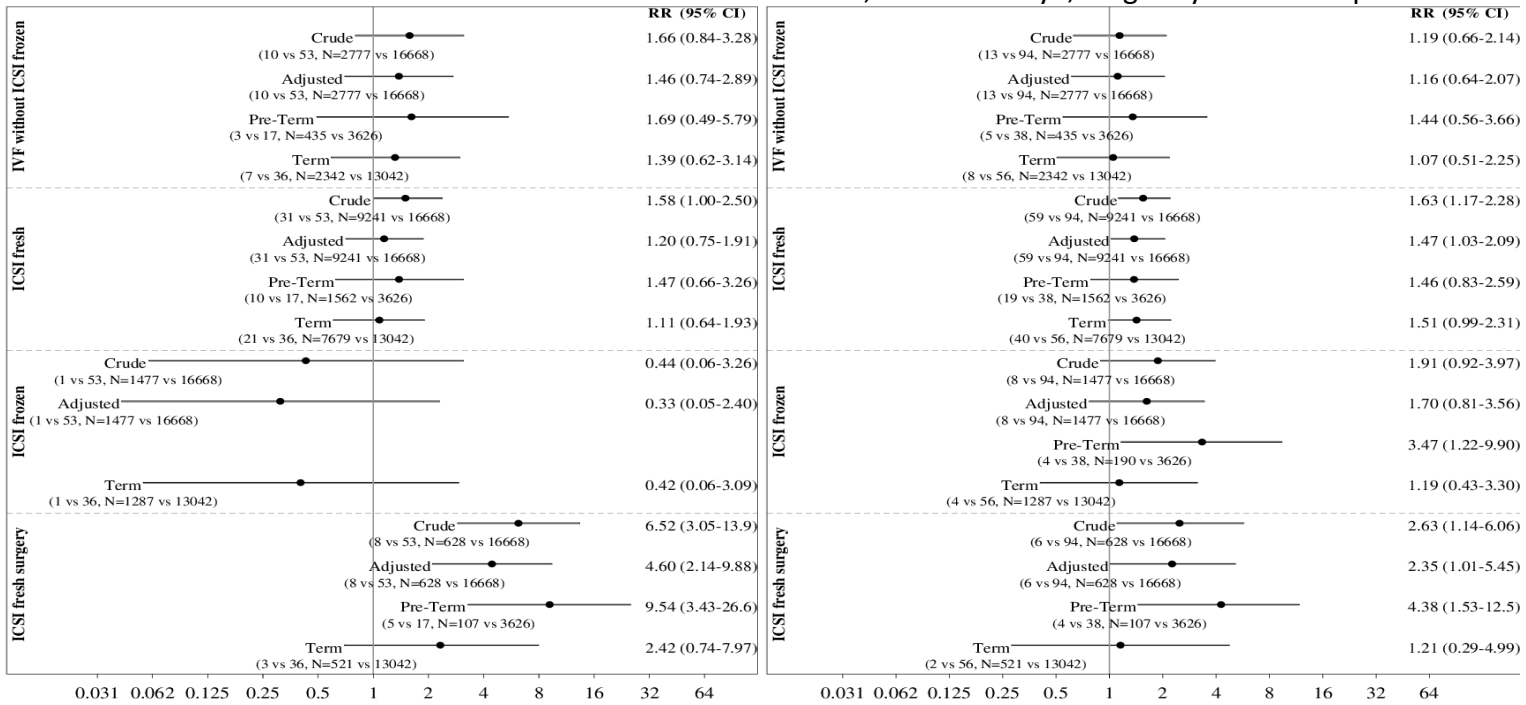
Comparing IVF procedures using blastocyst transfer with those using cleavage-stage transfer and comparing procedures using frozen embryos with those using fresh embryos, the risks for autistic disorder and mental retardation were not statistically significant.

Figure 1 **Comparing children born after any IVF vs Spontaneously conceived.** Relative risk of Autistic Disorder (left graph) and Mental Retardation (right graph) with spontaneously conceived as reference group. Dot for relative risk. Line bars for the two-sided 95% confidence intervals. All models except "Crude" are adjusted for confounding. Term and pre-term are subgroups of the data. Number of cases and number of children (N) in any IVF and reference group indicated within parenthesis under each line bar.



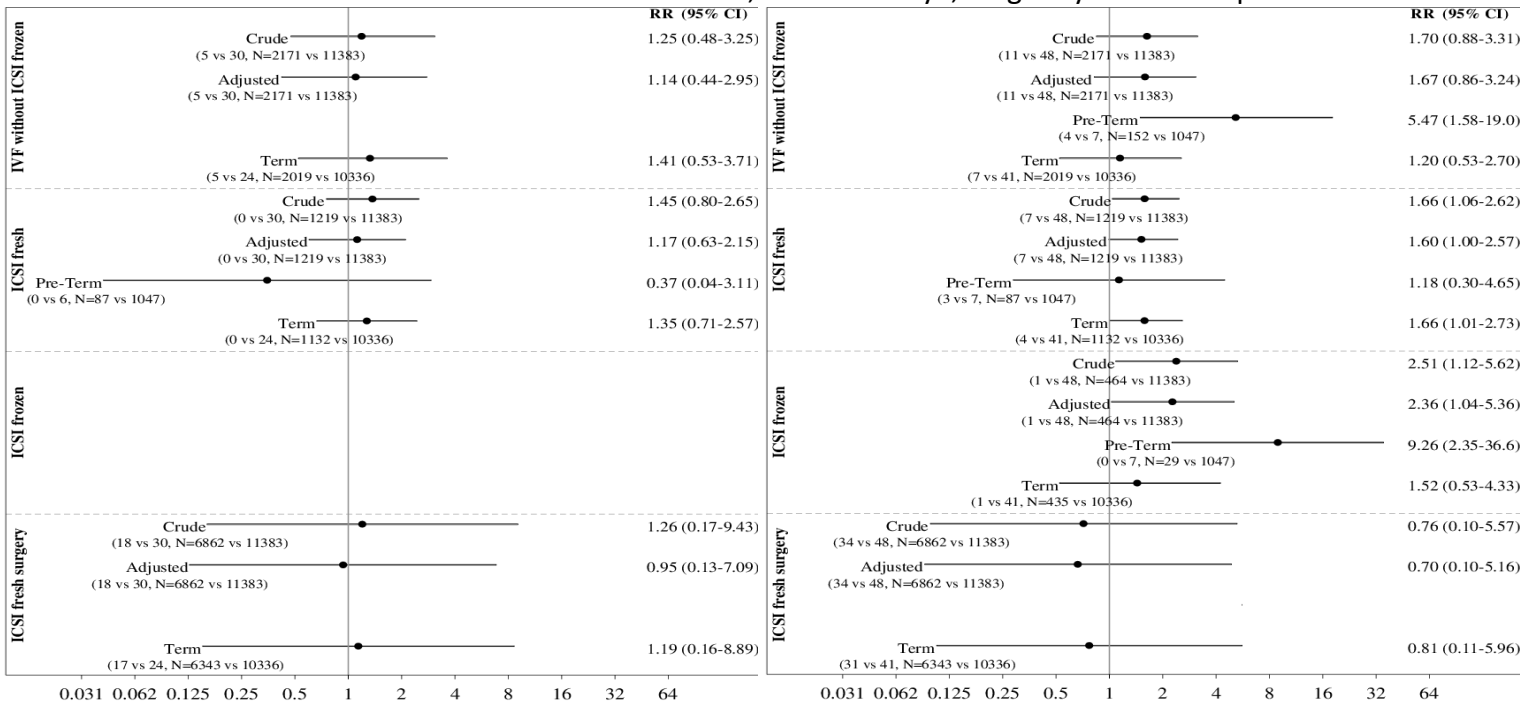
(xx vs xx, N=nn vs mm) indicate number of cases and number of children in the studied group vs reference group respectively. Crude: Adjusted for sex, attained age and birth year, Adj: Adjusted for sex, attained age, birth year, paternal age categorically, maternal age categorically, maternal psychiatric history at offspring birth (Y/N), paternal psychiatric history at offspring birth (Y/N)

Figure 2 **All children.** Relative risk of Autistic Disorder (left graph) and Mental Retardation (right graph) with IVF without ICSI, fresh embryo ejaculated sperm as reference. Dot for relative risk. Line bars for the two-sided 95% confidence intervals. All models except "Crude" are adjusted for confounding. Term and pre-term are subgroups of the data. Number of cases and number of children (N) in studied group and reference group indicated within parenthesis under each line bar. Groups in the figures without line bars do not have sufficient data to allow an estimation of the confidence intervals. For the same reason ICSI, frozen embryo, surgically extracted sperm is not shown.



(xx vs xx, N=nn vs mm) indicate number of cases and number of children in the studied group vs reference group respectively. Crude: Adjusted for sex, attained age and birth year, Adj: Adjusted for sex, attained age, birth year, paternal age categorically, maternal age categorically, maternal psychiatric history at offspring birth (Y/N), paternal psychiatric history at offspring birth (Y/N)

Figure 3 **Singletons.** Relative risk of Autistic Disorder (left graph) and Mental Retardation (right graph) with IVF without ICSI, fresh embryo ejaculated sperm as reference. Dot for relative risk. Line bars for the two-sided 95% confidence intervals. All models except "Crude" are adjusted for confounding. Term and pre-term are subgroups of the data. Number of cases and number of children (N) in studied group and reference group indicated within parenthesis under each line bar. Groups in the figures without line bars do not have sufficient data to allow an estimation of the confidence intervals. For the same reason ICSI, frozen embryo, surgically extracted sperm is not shown



(xx vs xx, N=nn vs mm) indicate number of cases and number of children in the studied group vs reference group respectively. Crude: Adjusted for sex, attained age and birth year, Adj: Adjusted for sex, attained age, birth year, paternal age categorically, maternal age categorically, maternal psychiatric history at offspring birth (Y/N), paternal psychiatric history at offspring birth (Y/N)

6.5 Supplementary analyses

The RR for specific IVF procedures using spontaneously conceived children as reference group were almost identical to the RR using IVF without ICSI fresh embryo as reference (Appendix B, eTable 10). The RRs did not change when adjusting for calendar year. When restricting to birth cohorts after 1998, the overall adjusted results remained stable and statistical significance remained except the risk for mental retardation following ICSI using surgically extracted sperm with fresh embryos, which dropped in precision RR, 2.08 ([95% CI: 0.74-5.89], rates 119.3 vs 61.2).

There were no major differences in risk of autistic disorder and mental retardation by age. The estimated RRs were similar in male and female offspring.

There was no increase in risk associated with years of infertility. Adjustment for this variable did not change the estimated associations with IVF/ICSI. The RR comparing hormone stimulation as the only treatment vs. spontaneous conception was not statistically significant different.

There were a total of 366 cases with known genetic diseases in the cohort, only 3 born following IVF (all following IVF with fresh embryos). Adjusting for presence of such conditions did not change the RR estimates or CIs.

For spontaneously conceived children multiple births contributed 2% of the person-years compared with 38% for IVF without ICSI with fresh embryos and 18-31% for other procedures. Among children with a diagnosis of autistic disorder or mental retardation, 3% were multiple births compared with 2% among children with no diagnosis at end of follow-up.

Among spontaneously conceived children, risk of autistic disorder in multiple births compared with singletons was RR, 1.15 ([0.95% CI: 0.99-1.34], rate 15.9 vs 13.8). Among children born after any IVF procedure RR, 1.88 ([95% CI:1.28-2.77], rates 46.0 vs 24.9) which was statistically significantly higher than among spontaneous conceived children (p=0.021) (Appendix B, eTable 11).

For mental retardation the comparable RR was RR 1.49 ([95% CI:1.11-2.00], rates 91.4 vs 60.0) among multiple births following IVF treatment and 1.42 ([95% CI:1.29-1.56], rates 51.1 vs 36.4) among spontaneously conceived multiples (Appendix B, eTable 11).

6.6 Discussion

Studies on long term neurodevelopment of children born following IVF treatment, especially after the first year of life, are limited. Studies on the association between IVF and autism (Maimburg & Vaeth 2006; Hvidtjørn et al. 2011; Lyall et al. 2012) or mental retardation (Strömberg et al. 2002; Carson et al. 2010; Leunens et al. 2008; Middelburg et al. 2008; Leslie et al. 2003; Bonduelle et al. 1998; Pinborg et al. 2004) show mixed results. A case-control study showed IVF to be a risk factor for autistic disorder (Zachor & Ben Itzchak 2011) while 2 other studies did not (Maimburg & Vaeth 2006; Hvidtjørn et al. 2011). Increased risk for developmental delay was reported in twins born following IVF (Strömberg et al. 2002), and in singletons following ICSI (Knoester et al. 2008), while a similar study did not find any difference (Ponjaert-Kristoffersen et al. 2005).

To the best of our knowledge this is the largest study examining the relationship between specific IVF procedures and autistic disorder and mental retardation, examining the full range of IVF procedures. While the data did not show an association between any IVF procedure and autistic disorder, compared with spontaneous conception, there was a small, statistically significant increase in the risk for mental retardation. When restricted to singletons, the risk for mental retardation was no longer statistically significant. However, the results demonstrated an association between autistic disorder and mental retardation and specific IVF procedures with ICSI related to paternal origin of infertility compared with IVF without ICSI.

The absolute differences in rates were small, below 7 per 100,000 person-years for mental retardation comparing any IVF procedure with spontaneous conception. While not a common treatment, the highest rate difference occurred with ICSI using surgically extracted sperm and fresh embryo transfer, compared with IVF without ICSI with fresh embryos (178.2 per 100,000 person-years for autistic disorder).

Our investigation of specific procedures was done in the subset of the population who all shared some degree of fertility problems. While this is the correct comparison for evaluating the effect of IVF beyond the general effects of sub-fertility, the question of how generalizable the data are can be raised (Carson et al. 2010). For this reason, we also presented these results using children born following spontaneous conception as

the comparison group (Appendix B, le 10).

Mental retardation was associated with ICSI with fresh embryos. This association was robust and not due to multiple births, premature birth, or parental characteristics. Mental retardation was also associated with ICSI with frozen embryos among children born prematurely (multiples or singletons), and with IVF without ICSI with frozen embryos among preterm singleton infants.

Autistic disorder and mental retardation were also associated with ICSI using surgically extracted sperm. The increase in risk was present in the analysis including all children and was stronger in preterm births. While the complete resolution of the risk in singletons can be explained by the reduction in statistical power it also suggest that the risk was, at least in part, mediated through multiple embryo transfer or preterm birth. In this context, the formal statistical analysis comparing multiples and singletons showed a higher rate of autistic disorder among multiples, although multiple birth may not be a risk factor for autistic disorder generally. An indirect cause for this might be the use of multiple embryo transfer in more severe cases of infertility or a direct effect of parental infertility factors.

We examined several alternative explanations for the results. First, hormone stimulation is part of IVF treatment. It has been suggested that use of hormones, not IVF treatment, is associated with increased risk of autistic disorder (Funderburk et al. 1983). We compared the risk of autistic disorder and mental retardation in children born to mothers reporting hormone treatment who had no IVF procedure. The risk for autistic disorder and mental retardation were not increased compared with the control population with RR point estimates below one.

Second, any risk associated with an IVF procedure could be due to advancing parental age or other parental characteristics. Adjusting for paternal and maternal age and for parental psychiatric history did not attenuate the risk associated with the IVF procedures.

Third, since 1981 the single-embryo transfers have increased from 10% to 70% of all treatments while the rate of premature births dropped from 40% to 10%. However, our results were not restricted to the earlier years of treatment and we adjusted for birth

year. Also, the RR remained unchanged in the sensitivity analyzes restricted to birth after 1998.

While we did not have information on the number of treatment cycles, there was no association with years of infertility. This association may however be complicated with different causes acting differently, e.g. if couples with paternal infertility tend to apply for IVF earlier.

A possible mechanism linking IVF and neurodevelopmental disorders is epigenetic modifications (Schanen 2006; Dada et al. 2012). Epigenetic processes have been associated with Rett's (Robertson & Wolffe 2000) and Angelman's syndroms (Mann & Bartolomei 1999), disorders characterized by autistic-like features in some patients. Experiments in mice have suggested that some of the steps involved in IVF might be related to epigenetic defects (Paoloni-Giacobino & Chaillet 2004; De Rycke et al. 2002). Mammal embryo cultured in-vitro is also susceptible to imprinting control (De Rycke et al. 2002). The risk of epigenetic changes may be modified the longer an embryo spends in culture. While blastocyst transfer is rare and also involves sperm selection, it offers an indirect test of this hypothesis. We did not find any change in risk with blastocyst transfer.

The strengths of this study include the large, prospective, population-based sample and a health system with equal access. We included more detailed IVF treatment information with longer follow-up and control for confounding than previously done. Closest in comparison is a cohort study of autistic spectrum disorder from 2011 (Hvidtjørn et al. 2011) that also included detailed control for confounding but only 9 years of follow-up, a sample size one fourth of ours, and no results on specific procedures. The detailed information allowed direct comparison of specific IVF procedures with IVF with fresh embryo transfer, allowing adjustment for shared confounding by causes of infertility and treatment.

The study have several limitations. We could not examine if multiple birth was associated with zygosity. We only had information on live births and cannot rule out confounding by miscarriage.

We did not have information on parental education or socioeconomic status. In

Sweden IVF treatment is free of charge for childless women for up to 3 treatment cycles. Additional cycles are not expensive compared with many other countries, but there are still many couples even in Sweden that cannot afford treatment beyond the 3 free-of-charge attempts. Any potential bias is likely to be small.

Information about the number of embryos transferred was only available from 2003. Therefore, this effect could not be reliably examined. The overall study objective of testing for an association between IVF/ICSI and autism or mental retardation is built from a composite hypothesis involving 10 statistical tests, of which 4 had unadjusted p-values below the 0.05 limit. After adjusting for multiplicity using Holm's procedure (Holm 1979), only 1 was statistically significant. Finally, some outcomes were based on small numbers, some estimates have wide confidence intervals and many others have lower confidence limits close to one. Future studies in different populations are needed to further examine these issues.

6.7 Conclusions

In Sweden, compared with spontaneous conception, any IVF treatment was not associated with autistic disorder but was associated with a small but statistically significantly increased risk of mental retardation. Regarding specific procedures, the use of IVF with ICSI for paternal infertility was associated with a small increase in the relative risk for autistic disorder and mental retardation compared with IVF without ICSI. The prevalence of these disorders was low, and the increase in absolute risk associated with IVF was small. These associations should be assessed in other populations.

Our results should be applicable to most countries where IVF and ICSI are used. There are no major differences in equipment or laboratory work across countries but there may be some differences in choice of procedure. For instance, in several countries (like the United States), ICSI is often used when the sperm sample is normal because of a presumed (but unproven) higher efficiency. Blastocyst transfer is infrequently used in Sweden but is more common in the United States.

7 Study IV - Familial risk of autism

This section contain the manuscript with the title "Familial risk of autism" as submitted to peer-review journals October 10, 2013.

7.1 Summary of the study

Importance

Autism Spectrum Disorders (ASD) is one of the most severe chronic development disorders. Studies have found that ASD aggregates in families, but to what extent this is caused by genetic factors, or shared or non-shared environment remains unresolved.

Objective

To provide estimates of familial aggregation and heritability of ASD.

Design, Setting and Participants

A prospective population based cohort of all children born in Sweden 1982-2007. Using national registers we identified all pairs of monozygote and dizygote twins, full siblings, maternal and paternal half siblings and cousin pairs. We included all clinical diagnosis of ASD to 31st December 2009.

Main Outcome Measures(s)

Familial aggregation of ASD and AD was evaluated by calculating relative recurrence risk for different family relations. Extended twin-models estimate how much of the probability of developing ASD can be attributed to genetic (additive and dominance) and environmental (shared and non-shared) factors.

Results

The ASD relative recurrence risk was estimated to 148.7 (95% Confidence Intervals (CI) 57.5 - 384.9) for monozygotic twins, 8.4 (95%CI 3.9-18.3) for dizygotic twins, 10.4 (95%CI 9.5-11.4) for full-siblings, 3.3 (95%CI 2.6-4.2) for maternal half siblings, 2.9 (95%CI 2.2-3.7) for paternal half siblings, and 2.0 (95%CI 1.8-2.2) for cousins. We found no statistically significant differences in recurrence risk according to sex. The recurrence risk pattern was similar for AD but of higher magnitude.

Extended twin-models supported a disease etiology including only additive genetic and

non-shared environmental effects components for both ASD and AD. The ASD heritability was estimated to 50% (95%CI 0.44-0.55) and the AD heritability was estimated to 0.54 (95%CI 0.44-0.64).

Conclusion and Relevance

In Sweden, genetic and environmental risk factors are equally important in ASD etiology. These results challenge the current dominant etiological model of ASD. The impact of familial risk factors can be quantified, providing a potential tool for family counseling.

7.2 Introduction

Autism Spectrum Disorders (ASD) are a group of neurodevelopmental disorders affecting almost 1% of the population, and defined by impairments in social interaction and communication and the presence of restricted interests and repetitive behaviors. Autistic disorder (AD) is most profound form of ASD (Hollander et al. 2010) .

Family studies have consistently found that ASD aggregates in families, suggesting a genetic component to the etiology. Early twin studies estimated the heritability of ASD, or the proportion of the phenotype variance due to genetic factors, to be about 90% (Bailey et al. 1995; Susan Folstein & Rutter 1977; Steffenburg et al. 1989; Lichtenstein et al. 2010; Ronald et al. 2006), making it the most heritable of all developmental disorders. As a consequence, etiological models and associated research in ASD, focus predominantly on genetic factors (Hallmayer et al. 2011). While recent twin studies support high heritability(Lichtenstein et al. 2010; Ronald et al. 2006) a large twin study (Hallmayer et al. 2011) indicated substantial role for shared environmental influences on risk for autism. Results of family studies also challenge the substantial role of genetic factors (Constantino et al. 2013). The mixed, sometime conflicting results, have brought considerable uncertainty regarding the etiology of ASD.

Furthermore, although previous studies were carefully conducted they have critical limitations. Concerns have been raised about twin studies often having only small samples limiting the reliability when estimating heritability of rare diseases such as ASD. None of the previous studies represent a prospective population based random sample which raises concerns for potential biases introduced by population selection. Restricted follow up time, , and possible differences in etiology for different ASD subtypes may also limit reliability. Furthermore, while heritability estimates provide a valuable metric for the effects of genetic factors in the population, they do not provide any information on individual risk. Detailed etiological models will require accounting for risk on a population level, as well as providing quantitative information in a given individual, thus allowing for individualized disease prevention and treatment(Manolio et al. 2009) .

Consequently, there is a need to obtain reliable estimates of heritability for ASD, as well

as combine these population-based estimates with individual-level risk estimates providing a more precise and complete picture of the etiology of ASD.

To that goal we conducted a prospective longitudinal cohort study of all births in Sweden between 1982 and 2007. Using all pairs of monozygote (MZ) and dizygote (DZ) twins, full siblings, half siblings and cousin pairs in the population we determined the family clustering of ASD by estimating relative recurrence risk within families, and assessed the importance of genetic vs. environmental factors in the etiology of ASD.

7.3 Methods

7.3.1 Study Population

A birth-cohort of all children born alive in Sweden January 1, 1982 to December 31, 2006 was established using data from Swedish national registers including the Medical Birth Register(Axelsson 2003) , Multi Generation Register(Ekbom 2011) , Patient Register(Ludvigsson et al. 2011; Sellgren et al. 2011; Ekholm et al. 2005) , Twin register(Lichtenstein, Sullivan, et al. 2006) and Statistics Sweden registers for vital statistics. All Swedish live-born children are assigned a unique personal identification number. The number is used in all contacts with authorities and ensuring accurate and complete individual-level linkage between registries. We defined the recurrence risk as the risk of autism following an autism diagnosis in a sibling (see Statistical methods), therefore single-child families were excluded from the cohort. Twin zygosity information was available from the Twin Registry. The study was approved by the ethics committee at the Karolinska Institutet, Stockholm, Sweden.

7.3.2 Ascertainment of autism and psychiatric diagnosis

In Sweden all infants and preschool children are regularly seen at well-child care clinics and undergo routine medical and developmental screening. At age 4 a mandatory developmental assessment (motor, language, cognitive and social development) is conducted. Children with suspected developmental disorders are referred for further assessment by a specialized team in a child psychiatry unit or habilitation service. Diagnostic information is reported to the Patient Register. The register has nearly complete national coverage (Ludvigsson et al. 2011) and include psychiatric in-patient diagnoses since 1973 and out-patient diagnoses from the year 2000. With prospective

follow-up until 31st December, 2009. Autistic disorder (AD) was defined by ICD-9 299.A/B/X and ICD-10 F84.0 while ASD also included ICD-10 F84.1 (Atypical autism), F84.5 (Asperger's syndrome), F84.8 (Other pervasive developmental disorders) and F849 (Pervasive developmental disorder, unspecified).

7.3.3 Covariates

We considered several factors that might confound or modify the familial associations. Parental psychiatric history was classified as present/not-present for each parent separately using any psychiatric diagnosis at any time before the birth of the oldest child in a siblings or cousins pair using ICD 7th-10th revisions. We also obtained information on parental age, birth year and sex.

7.3.4 Statistical methods

The relative recurrence risk (RR) for siblings is the risk of autism diagnosis in a sibling of an autistic child compared with a sibling to a non-autistic child. We calculated recurrence risk in families of different genetic relatedness; full-siblings, maternal and paternal half-siblings and cousins. Cousin-pairs were defined as cohort members having the same grandparents, but no parents in common. To allow a direct comparison between cousin recurrence risk and sibling recurrence risk we did not consider cousins between single-child-families.

We estimated the RR for ASD by the hazard ratios obtained from Cox proportional hazards regression using the sibling attained age as underlying time scale(Korn et al. 1997). When the exposure is not an affected family member the recurrence calculated here is commonly labeled as Relative Risk (RR). Each individual in a sibling or cousin pair was entered into the cohort and followed for a diagnosis of autism starting from the age of one or from 1st of January 1987, which ever came first. Each sibling/cousin was then followed to his first autism diagnosis, death, emigration or death or emigration of his non-autistic sibling or 31 December 2009, whichever came first. The exposure (autistic or non-autistic sibling) was treated as a time-varying covariate in the models. As each sibling in a sibling pair can contribute to the calculations twice (both as an exposed sibling and as a proband) we used robust standard errors(Liang & Zeger 1986).

For descriptive purposes we calculated the cumulative probability of ASD up to the age of 20 using the Cox regression. For the calculation of relative recurrence risk the Cox regression makes an implicit assumption of hazards ratios constant across time (age of the sibling). We verified the validity of this assumption by plotting the Schoenfeld residuals(Grambsch & Therneau 1994) .

A change in RR for later birth cohorts may be due to truncation of follow-up time or due to changes in incidence. The children born 1982 are followed for 28 years while the children born 2006 are only followed for three years. In the Cox model this could show up as a violation of the proportional hazards assumption which we tested for. To address this further we calculated the RR by birth cohorts using all available follow-up time.

We excluded multiple births from the sibling analyzes. We calculated the RR separately for monozygotic and dizygotic twins, full siblings, maternal and paternal half siblings as well as for cousins. We considered several factors that might confound the recurrence risk including parental psychiatric history, parental age, birth year and sex of the exposing sibling. As parental psychiatric history and parental age may be on a causal path between familial risk and adverse developmental outcome we fitted models adjusting for confounding with and without these covariates. We treated the covariates categorically as sex of the exposed sibling, birth cohort (1982-86, 1987-91, 1992-96, 1997-2001, 2002-06), maternal age (≤35, >35), paternal age (≤40, >40), and paternal and maternal psychiatric history (yes/no) at birth of the oldest sibling.

We used an extended sibling design (MZ twins, DZ twins, full siblings, maternal half siblings, paternal half siblings) to decompose the variance in liability into additive genetic factors (A) reflecting additive effects of different alleles, non-additive genetic factors (dominance, D) reflecting interaction effects between alleles at the same gene locus, shared environmental factors (C) reflecting non-genetic influences that contribute to similarity within pairs of siblings and non-shared environmental factors (E) reflecting experiences that make sibling pairs dissimilar. MZ twins are assumed to share 100% of their *A* in a pair and DZ twins are assumed to share 50% of their *A*; full siblings, assumed to share 50%, maternal half siblings, assumed to share 25%, and paternal half siblings, who are assumed to share 25% of *A* in a pair. Furthermore we

assume that all sibling types included share the *C*-parameter in pairs, except paternal half siblings since children tend to be living with their mother while growing up(Moeller 1994). We also included dominant genetic effects (*D*), 100% shared between MZ twins, 25% between DZ twins, 25% between full siblings, and not shared between half siblings. We first calculated tetrachoric correlations. Contrasting the tetrachoric correlations for different family relations allowed for an initial examination of the relative contribution of genetic and environmental influences. We then estimated the relative contribution of genetic (i.e., A and D) and environmental (i.e., C and E) using the liability-threshold model where an underlying normal distribution is assumed for the liability of having the disease (Neale & Cardon 1992, pp.43–77 pp43-77). Including the five different types of siblings made the model identifiable. We allowed for different prevalence of the outcome in the different sibling types, and adjusted the prevalence for gender and birth cohort.

First we compared the ACE, ADE and DCE models with the full ADCE model. Next, using likelihood ratio tests, submodels where the genetic parameter, shared environmental parameter, and both these parameters are dropped (AE, DE,CE, and E models), were tested to explain the observed data and pattern of variance using as few parameters as possible. For comparison purposes we calculated heritability among twins only using the tetrachoric correlations as well.

All calculations were done for ASD and AD separately. All tests of statistical hypothesis were done on the two-sided 5% level of significance. We used SAS software version 9.3 and the R software version 2.15.2 Linux 64-bit (survival package for Cox regression with robust standard errors). For heritability analyses we used R software version 2.15.2 Windows 32-bit (OpenMx package version 1.3.1-2179)(Boker et al. 2011) .

7.4 Results

Cohort characteristics are presented in Table 1. The cohort included 2,711,265 full sibling pairs, 442,408 maternal half sibling pairs, 455,219 paternal half sibling pairs and 37,805 twins and 11,643,186 cousin pairs. We found 14,524 cases of ASD of which 5,687 (39%) had a diagnosis of AD.

Table 1 Confounder and baseline characteristics distributions across sibling relations

Variable	Full Siblings	Maternal Half Siblings	Paternal Half Siblings	Cousins	DZ Twins	MZ Twins
Subject pairs	2,711,265	442,408	455,219	11,643,186	29,424	8,381
Subjects	1,819,174	292,739	290,748	1,241,226	29,032	8,338
Boys (%)	51.4%	51.2%	51.1%	51.5%	51.0%	47.2%
ASD cases (%)	12,276 (0.67%)	2,981 (1.02%)	2,578 (0.89%)	8,073 (0.65%)	215 (0.74%)	41 (0.49%)
AD cases (%)	4,891 (0.27%)	1,037 (0.35%)	925 (0.32%)	3,021 (0.24%)	97 (0.33%)	21 (0.25%)
Maternal Psych. History (%)[#]	40,221 (2.21%)	18,724 (6.40%)	14,690 (5.05%)	25,180 (2.03%)	908 (3.13%)	196 (2.35%)
Paternal Psych. History (%)[#]	39,262 (2.16%)	15,909 (5.44%)	16,373 (5.63%)	23,778 (1.92%)	792 (2.73%)	200 (2.40%)
Birth Year, Median (p5-p95)	1993 (1984-2005)	1993 (1983-2005)	1993 (1983-2005)	1993 (1984-2004)	1996 (1983-2005)	1994 (1982-2002)
Age at ASD diagnosis, Median (p5-p95)	12 (4-22)	13 (4-22)	13 (4-23)	13 (4-22)	11 (4-21)	10 (4-25)
Person Years, Median (p5-p95)	14 (4-24)	11 (3-21)	10 (3-21)	15 (4-24)	13 (3-25)	14 (7-26)

ASD: Autism Spectrum Disorder; AD: Autistic Disorder (infantile autism); p5: 5th percentile, p95: 95th percentile. #: At birth of the child

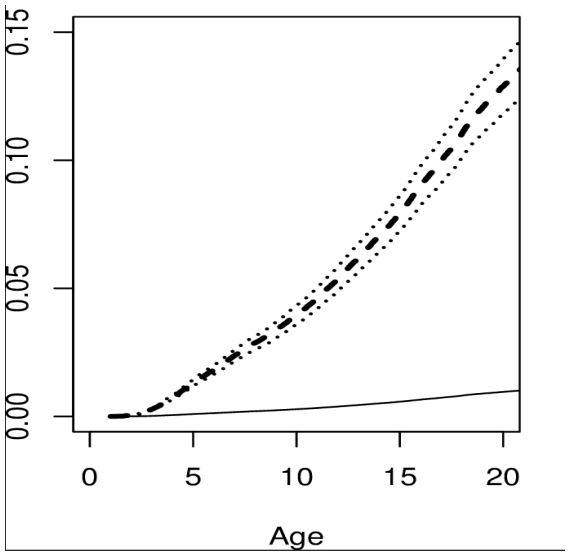
eTable 4 Number of births, ASD and AD cases and prevalences by year of birth

Birth Year	Number of Births	ASD Cases	ASD Prevalence (%)	AD Cases	AD Prevalence (%)
82-86	321,206	1,961	0.61	448	0.14
87-91	503,960	4,377	0.87	1321	0.26
92-96	489,652	4,506	0.92	1790	0.37
97-01	389,999	2,606	0.67	1388	0.36
02-06	348,650	1,074	0.31	740	0.21

ASD: Autism Spectrum Disorder; AD: Autistic Disorder (infantile autism); p5: 5th percentile, p95: 95th percentile

The ASD observed crude prevalence varied between 0.31% to 0.92% during the 5-year-band birth cohorts 1982-2006 (eTable 4). For individuals with a sibling with ASD the cumulative probability of an ASD diagnosis at age 20 was estimated to 13% compared with 1.2% for individuals without an ASD sibling (figure 1).

Figure 1 Age-cumulative probabilities for ASD diagnosis in sibling with and without a sibling with an earlier ASD diagnosis. 95% two-sided point wise confidence bands for exposed siblings.

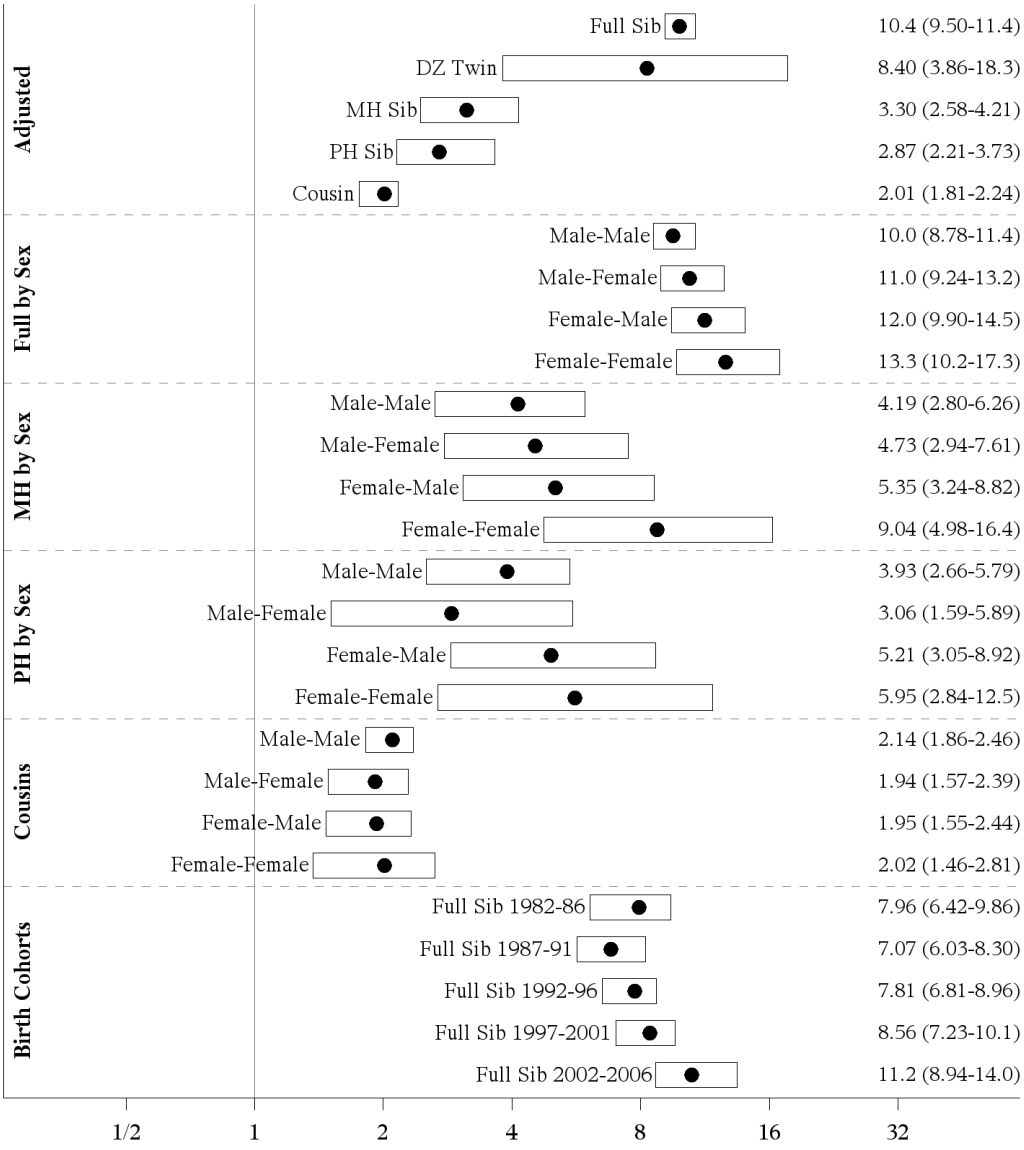


Dashed line: Cumulative probability of an autism diagnosis up to this age for siblings with a sibling proband with an autism diagnosis. Solid line: Cumulative probability of an autism diagnosis up to this age for siblings with a sibling proband free from an autism diagnosis.

7.4.1 Relative recurrence risk

Figure 2 presents relative recurrence risks for ASD and associated two-sided 95% confidence intervals for the different degrees of genetic distance between family relatives. The RR remained stable after adjustment for sex, parental psychiatric history and parental age. There was some support for confounding attributable to birth cohorts (figure 2, bottom panel).

Figure 2 ASD recurrence risks for full and maternal (MH) and paternal (PH) half siblings, cousins and DZ twins. Point estimates and two-sided 95% confidence intervals. MZ twins not shown.



Footnote: Male-Female indicate risk in female exposed to a male relative. The MZ adjusted RR was 148.7 (95% CI 57.5 - 384.9), outside the range of the figure. Adjusted: Models adjusting for birth cohort and sibling and proband sex and paternal and maternal psychiatric history at birth of the child and older maternal age (≤ 35, > 35) and older paternal age (≤ 40, > 40); MH: Maternal half siblings, PH: Paternal half siblings; Old Pa: Paternal age > 40; Yng Pa: Paternal age ≤ 40; Old Ma: Maternal age > 35; Yng Ma: Maternal age ≤ 35; Fa Psych; With a paternal psychiatric history; Fa Psych: With a paternal psychiatric history; With a maternal psychiatric history; Ma Psych: With a maternal psychiatric history.

When adjusting for 5-year birth cohorts, sex of child and proband and parental and maternal psychiatric history the RR was 148.7 (95%CI 57.5 - 384.9) for monozygotic twins, 8.4 (95%CI 3.9 - 18.3) for dizygotic twins, 10.4 (95%CI9.5 - 11.4) for full siblings, 3.3 (95%CI 2.6 - 4.2) for maternal half siblings,2.9 (95%CI 2.2 - 3.7) for paternal half siblings and 2.0 (95%CI 1.8 - 2.2) for cousins. For crude RR see eTable 3.

eTable 3 Crude (no adjustment for confounding) recurrence risk (RR) and two-sided 95% confidence intervals

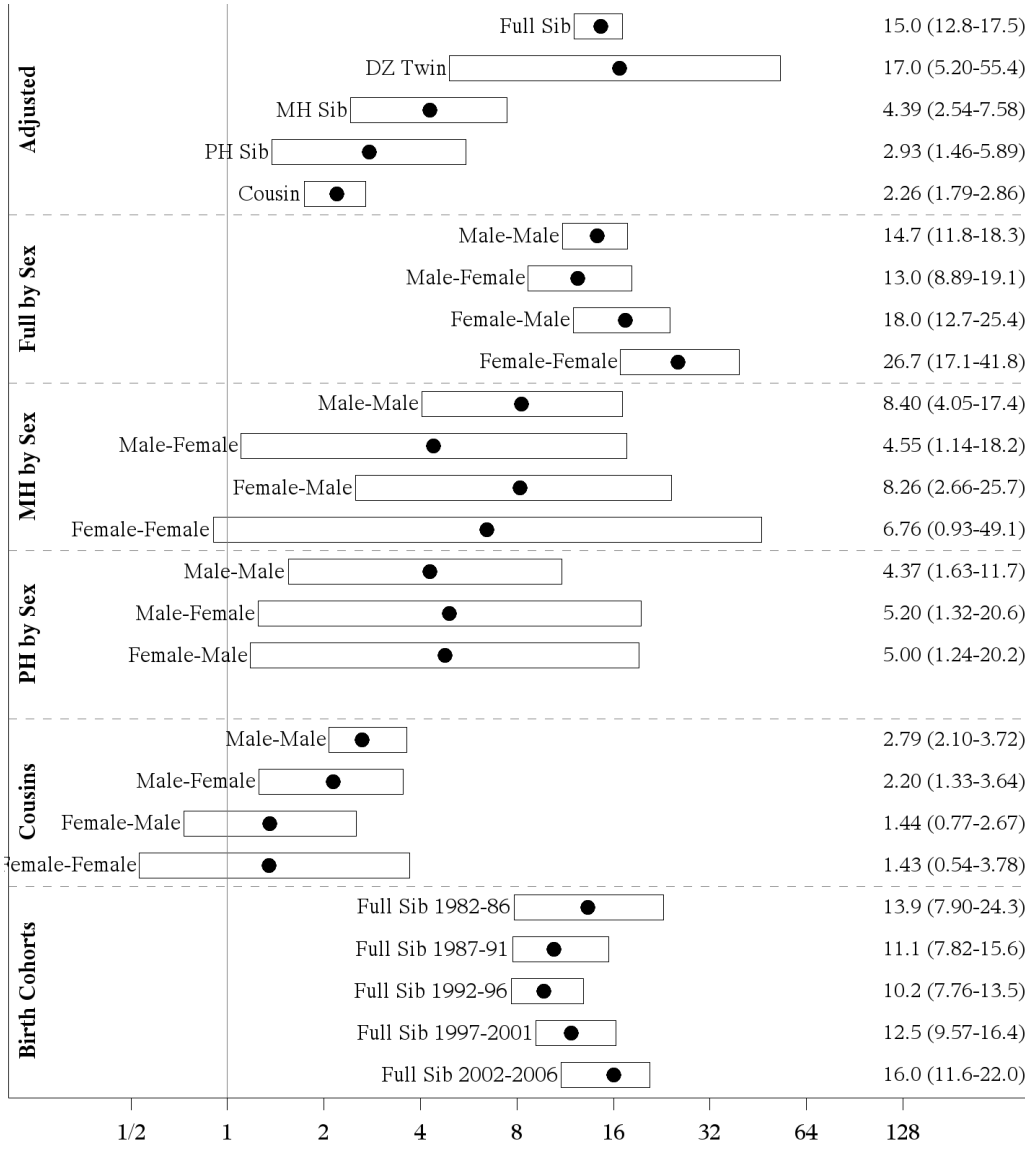
Family relation	**ASD**		**AD**	
	Pairs	**RR (95% CI)**	**Pairs**	**RR (95% CI)**
MZ Twins	41	217.06 (85.22 - 552.86)	21	301.76 (57.87 - 1573.38)
DZ Twins	217	12.38 (5.65 - 27.12)	99	28.78 (8.75 - 94.64)
Full Siblings	17,961	14.35 (13.10 - 15.72)	7,273	24.18 (20.66 - 28.30)
Paternal Half Siblings	3,999	4.24 (3.26 - 5.51)	1,462	4.32 (2.17 - 8.63)
Maternal Half Siblings	4,446	4.98 (3.91 - 6.35)	1,573	7.60 (4.43 - 13.06)
Cousins	72,436	2.723 (2.45 - 3.025)	27,352	3.35 (2.65 - 4.24)

MZ: Monozygotic; DZ: Dizygotic; ASD: Autism Spectrum Disorder; AD: Autistic Disorder

RR for AD are presented in figure 3. The RR for AD had a generally higher magnitude compared with ASD but with lower prevalence. Adjusting for 5-year birth cohorts, sex of child and proband and parental and maternal psychiatric history the RR was 116.8 (95%CI 16.7 - 814.2) for monozygotic twins, 17.0 (95%CI 5.2-55.4) for dizygotic twins, 15.0 (95%CI 12.8-17.5) for full sibling 4.4 (95%CI 2.5-7.6) for maternal half siblings,2.9 (95%CI 1.5-5.9) for paternal half siblings and2.3 (95%CI 1.8-2.9) for cousins.

While graphical inspection of the RR point estimates suggest a trend for higher RR in female-female sibling pairs there were no statistically significant differences in RR between males and females or by sex of the proband (figure 2, figure 3). The model goodness-of-fit supported the assumption of hazards being proportional over the time of follow-up.

Figure 3 AD recurrence risks for full and maternal (MH) and paternal (PH) half siblings, cousins and DZ twins. Point estimates and two-sided 95% confidence intervals. MZ twins not shown.



Footnote: Male-Female indicate risk in female exposed to a male relative. The MZ adjusted RR was 116.8 (95% CI 16.7 - 814.2), outside the range of the figure. Adjusted: Models adjusting for birth cohort and sibling and proband sex and paternal and maternal psychiatric history at birth of the child and older maternal age (≤ 35, > 35) and older paternal age (≤ 40, > 40); MH: Maternal half siblings, PH: Paternal half siblings; Old Pa: Paternal age > 40; Yng Pa: Paternal age ≤ 40; Old Ma: Maternal age > 35; Yng Ma: Maternal age ≤ 35; Fa Psych; With a paternal psychiatric history; Fa Psych: With a paternal psychiatric history; With a maternal psychiatric history; Ma Psych: With a maternal psychiatric history

7.4.2 Heritability

The unadjusted ASD tetrachoric correlation was estimated to 0.54 (SD=0.203) for MZ twins; 0.25 (SD=0.127) for DZ twins; 0.25 (SD=0.015) for full siblings; 0.11 (SD=0.039) for maternal half siblings and to 0.07 (SD=0.049) for paternal half siblings; (eTable1). For AD the tetrachoric correlation for full siblings was estimated to 0.27 (SD=0.025); for maternal half siblings to 0.13 (SD=0.079) and for paternal half siblings 0.14 (SD=0.078) while MZ and DZ twins did not allow estimation due to sample size (eTable2). The tetrachoric correlations adjusted for sex and birth cohort were almost identical (eTable1, eTable 2).

eTable 1 ASD. Tetrachoric correlations (SD)

Sibling Relation	Un-adjusted					Adjusted for sex and birth cohort
	Both genders	Female	Male	Female-Male	Male-Female	Both genders
All	0.22 (0.012)	0.23 (0.035)	0.22 (0.021)	0.22 (0.026)	0.24 (0.029)	
MZ	0.54 (0.203)	x	0.70 (0.203)	x	x	0.52 (0.214)
DZ	0.25 (0.127)	x	x	0.38 (0.193)	0.33 (0.200)	0.26 (0.130)
Full Siblings	0.25 (0.015)	0.25 (0.041)	0.25 (0.024)	0.24 (0.029)	0.29 (0.032)	0.25 (0.015)
MH Siblings	0.11 (0.039)	0.25 (0.088)	0.06 (0.068)	0.13 (0.075)	0.10 (0.093)	0.13 (0.040)
PH Siblings	0.07 (0.044)	0.05 (0.132)	0.13 (0.063)	0.09 (0.087)	x	0.08 (0.045)

MZ: Monozygotic twins; DZ: Dizygotic twins; x: Not estimable (missing observation where both siblings are cases)

eTable 2 AD. Tetrachoric correlations (SD)

Sibling Relation	Un-adjusted					Adjusted for sex and birth cohort
	Both genders	Female	Male	Female-Male	Male-Female	Both genders
All	0.24 (0.023)	0.2715 (0.0628)	0.27 (0.034)	0.26 (0.045)	0.16 (0.066)	
MZ	x	x	x	Not applicable	Not applicable	x
DZ	x	x	x	x	x	x
Full Siblings	0.27 (0.025)	0.3238 (0.0657)	0.30 (0.037)	0.26 (0.052)	0.17 (0.076)	0.28 (0.025)
MH Siblings	0.131 (0.079)	x	0.08 (0.136)	0.283 (0.115)	x	0.15 (0.081)
PH Siblings	0.14 (0.078)	x	0.10 (0.136)	0.19 (0.142)	0.21 (0.148)	0.14 (0.080)

MZ: Monozygotic twins; DZ: Dizygotic twins; x: Not estimable (missing observation where both siblings are cases)

Since the full ACE and ADE models gave similar fits the ACE model was chosen as the full model under which nested sub-models were tested. Using likelihood ratio tests where models with fewer parameters were compared with the full ACE model the best fitting model was the AE model, that is, a model with only a genetic (heritability) and a non-shared environment component (Table 2). On the basis of the AE model the ASD heritability was estimated to $h^2 = 0.50$ (95%CI 0.46-0.56) and the non-shared environmental influence was 0.50 (95%CI 0.44-0.55).

In the full ACE model, also including the shared environment, the variance associated with the shared environment was estimated to 0.04 (95%CI 0-0.15), non-shared environment to 0.54 (95%CI 0.44-0.66) and heritability to 0.42 (95%CI 0.19-0.55). Using twins only the heritability was estimated to 0.52.

For AD the AE model was the best fitting model as well (Table 2) and the AD heritability was estimated to $h^2 = 0.54$ (95%CI 0.44-0.64).

Table 2 ASD and AD Heritability. Model goodness of fit and variance component estimates.

Model	#p	-2 LL	Diff -2 LL	p-value	Variance components (95% Confidence Intervals)				$a^2 + d^2$
					a^2 (heritability)	d^2	c^2	e^2	
ASD - Autism Spectrum Disorder									
ADCE	14	143,909.8	NA	NA	0.33 (0.00-0.55)	0.16 (0.00-0.59)	0.05 (0.00-0.17)	0.46 (0.24-0.65)	0.49 (0.21-0.75)
ACE	13	143,910.4	0.7	0.415	0.42 (0.19-0.55)	x	0.04 (0.00-0.15)	0.54 (0.45-0.66)	0.42 (0.19-0.55)
ADE	13	143,910.53	0.8	0.382	0.44 (0.24-0.55)	0.13 (0.00-0.51)	x	0.43 (0.23-0.55)	0.57 (0.45-0.77)
DCE	13	143,912.8	3.0	0.082	x	0.45 (0.18-0.71)	0.14 (0.07-0.20)	0.41 (0.21-0.62)	0.45 (0.18-0.71)
AE	12	143,911.0	1.2	0.550	0.50 (0.45-0.56)	x	x	0.50 (0.44-0.55)	0.50 (0.45-0.56)
DE	12	143,933.6	23.8	0.000	x	1.00 (1.00-1.00)	x	0.00 (0.00-0.00)	1.00 (1.00-1.00)
CE	12	143,923.1	13.3	0.001	x	x	0.24 (0.21-0.26)	0.76 (0.73-0.79)	x
E	11	144,178.5	268.8	0.000	x	x	x	1.00 (1.00-1.00)	x
AD - Autistic Disorder									
ADCE	14	64,586.2	x	x	0.49 (0.00-0.64)	0.00 (0.00-0.61)	0.02 (0.00-0.24)	0.48 (0.18-0.72)	0.49 (0.04-0.82)
ACE	13	64,586.2	0.0	0.992	0.49 (0.04-0.64)	x	0.03 (0.00-0.24)	0.48 (0.36-0.72)	0.49 (0.04-0.64)
ADE	13	64,586.3	0.1	0.807	0.54 (0.25-0.64)	0.00 (0.00-0.54)	x	0.46 (0.17-0.56)	0.54 (0.44-0.83)
DCE	13	64,590.7	4.5	0.034	x	0.65 (0.00-0.84)	0.11 (0.04-0.30)	0.23 (0.10-0.79)	0.65 (0.00-0.84)
AE	12	64,586.3	0.1	0.971	0.54 (0.44-0.64)	x	x	0.46 (0.36-0.55)	0.54 (0.44-0.64)
DE	12	64,645.6	59.4	0.000	x	1.00 (1.00-1.00)	x	0.00 (0.00-0.00)	1.00 (1.00-1.00)
CE	12	64,590.9	4.7	0.096	x	x	0.26 (0.21-0.31)	0.74 (0.69-0.79)	x
E	11	64,682.9	96.8	0.000	x	x	x	1.00 (1.00-1.00)	x

#p: Number of parameters in the model; -2 LL: -2 * log-likelihood; Diff df: Number of degrees of freedom for the -2 LL (difference in number of parameters between the model and the ADCE model); Diff -2 LL: 2 * difference in log-likelihood between the model and the ADCE model; p-value: p-value for the testing the hypothesis the parameters not in the model but in the ADCE model are all equal to zero; a^2: Additive genetic; d^2: Dominant genetic; c^2: Shared environment; d^2: Non-shared environment; $a^2 + d^2$: "Broad-sense heritability" including both the additive and the dominant genetic components. x: Not applicable. Note: All models adjusted for gender and birth cohort.

7.5 Discussion

To the best of our knowledge this is the largest population based longitudinal study evaluating familial risk and heritability of ASD. Studying more than 2 million Swedish families documented the familial aggregation of ASD across the range of genetic relations to a proband showing increased relative risk with increasing genetic relatedness. Using the family data we found evidence for similar importance for genetic and environmental influences on liability for ASD. The results were similar for ASD and AD.

Heritability of ASD was estimated to 50%, suggesting that genetic factors explain half of the liability for autism. 50% of the liability for ASD was related to unique environmental factors. i.e. to experiences that are different between members of the same family. These results should be replicated in other populations, yet they provide strong evidence for an equal role for environmental and genetic factors in the etiology of ASD.

This estimate of heritability is considerably lower than the 90% in earlier twin studies (Bailey et al. 1995; Susan Folstein & Rutter 1977; Steffenburg et al. 1989) and closer to that of a recent California twin study which estimated that the heritability of ASD was 38%(Hallmayer et al. 2011) . The heritability estimate can also be compared with a Swedish twin cohort (Ronald et al. 2011) of more than 12,000 children where heritability of between 49% and 72% was reported for autistic-like traits (social impairment, communication impairment and restricted and repetitive behavior and interests).

Earlier twin studies documented only minimal non-shared environmental contribution to liability to ASD. The California twin study, in contrast, suggested substantial shared environmental influences, i.e. to experiences that are common between members of the same family. The large family data in our study indicated that such influences have only a negligible effect on ASD etiology. Dizygotic twins and full siblings (both having 50% genetic similarity, but dizygotic twins assumed to have more shared maternal prenatal environment), and maternal half siblings and paternal half siblings (both having 25% genetic similarity, but maternal half siblings assumed to have more shared

maternal prenatal environment) had comparable risks for ASD. In the presence of a familial confounding, factors effecting all members of a family, the RR is expected to be lower for the dizygotic twin compared with full siblings and for the maternal half-siblings compared with the paternal half-siblings. The RR can also be compared with the RR for schizophrenia, a neurodevelopmental disorder with earlier overlap in diagnosis and with shared clinical an etiological features (Stone & Iguchi 2011). In a sample overlapping with the parents and grandparents of our study the RR was estimated to 8.5 for full siblings, 2.5 for half siblings and 2.3 for cousins (Lichtenstein, Björk, et al. 2006).

The differences in results between the present study and earlier research may in part be attributed to differences in sampling, case ascertainment and analytic approach. The present study used a true population based sample, continuously following up participants since birth and previously validated clinical diagnosis of ASD done by expert clinicians. Previous twin studies relied on considerably less robust methodologies for case ascertainment, including self-referral, service registers, and parental reports on diagnosis. Even when detailed diagnostic assessment was done the participation rates were low and it could not be ruled out that participation was associated with presence of an autistic child in the family (Bailey et al. 1995), limiting generalizability. We adjusted for birth cohorts, trying to address potential biases due to differences in length of follow-up with study subjects in different birth years (Lindström et al. 2006). It is unclear how this was addressed and effected previous studies. We believe the effect of such a bias could inflate the shared environment component.

Factors potentially effecting the variance for non-shared environment includes a misclassification of cases. This could further be supported with possible, but unknown, differences in etiology across the different forms and symptoms of ASD symptoms. Our data do not support this though as our results for the liability of ASD and AD were essentially the same.

The relative recurrence risk between different pairs of family members reflects the genetic influences on the trait and offers a quantitative and practical measure of familial risk. Thus, the relative recurrence risk has an important application which distinguishes it from the more theoretical measures of heritability. For example while

genetic factors account for 50% of individual differences in liability to ASD, a sibling of a proband with ASD who shares 50% of the genes has a 10-fold increase in risk. This can potentially be applied at an individual level for family counseling.

Only few earlier studies have had the possibility to calculate the relative recurrence risk (Grønborg et al. 2013; Ritvo et al. 1989; Constantino et al. 2013). Two studies are presenting self-selected samples (Constantino et al. 2013; Ritvo et al. 1989) and with limited family data. A recent Danish study provide reliable estimates using an excellent epidemiological sample similar to ours. They show lower RR, RR=7.5 for full siblings but with similar relative relation between full siblings and maternal and paternal half-siblings. Our sample include twice as many cases of ASD and more detailed family data including monozygotic and dizygotic twins and cousins. Our bigger sample also allowed us to investigate sex of offspring in some more detail. Several earlier studies have reported absolute sibling recurrence risk (Ritvo et al. 1989; Szatmari et al. 1998; Bolton et al. 1994; Chudley et al. 1998; Sumi et al. 2006; Constantino et al. 2010; Ozonoff et al. 2011) but absolute risk is a cumulative measure which depends on the length of follow up (higher at age 15 than at age 5) and will differ between populations. As elsewhere in epidemiology, where the relative risk is a preferred measure of disease risk, the relative recurrence risk circumvent these limitations.

This study has multiple strengths including the large, prospective, full-nation population-based sample and a health system with equal access. In addition to sibling pairs we were also able to include cousins and twins including zygosity information and to adjust for parental psychiatric history. To estimate the RR we used time-to-event methods to avoid introduction of bias due to differences in follow-up time for different subjects. The methods of analyzing risk between siblings will also adjust for potential bias due to changes in prevalence of autism in later years where later born siblings may be expected to have a higher risk of being diagnosed.

The same underlying population was used to estimate the recurrence risk and heritability. We are using a prospective cohort approach for the sampling and following all subjects from birth and onwards using clinical registers. By utilizing this approach we are avoiding potential and unpredictable selection-biases due to disease status or factors such as parental education. Using the registers we are also avoiding problems

associated with self-reports and retrospective collection of data. We included birth year in the statistical models estimating heritability to avoid biases due to differences in length of follow-up and due to confounding by temporal trend.

The study has some limitations. We did not have information on parental education or socioeconomic status. In Sweden there is equal access to health services and they are free of charge. Any potential bias is therefore likely to be small. We also did not have information on level of intellectual disability in ASD. This may be studied separately in future studies. There is a well documented gender bias in autism (Fombonne 2005), and it has been suggested that females may require greater familial etiologic load to manifest the autistic phenotype (Robinson et al. 2013). We did not find strong support for any sex specific differences in the relative recurrence risk. This effect might be small, and therefore require even greater samples to be reliably documented. The most important confounder was birth year. The RR was slightly increased over the last few birth cohorts (figure 2, figure 3). This could probably be explained by the increase in prevalence in the later years. Relatives born closer in time have a more similar base probability for ASD than relatives born distal in time. Due to power limitations the adjustment for birth year was only possible by assuming a strict linear relation for the MZ twins while these estimates may be slightly more biased.

7.6 Conclusion

The results of this study show equal importance for genetic and environmental influences on the risk of ASD. These results challenge current etiological models of ASD, which weights towards greater influence of genetic factors. This study can also have clinical implications: The risk information for twins, siblings and cousins should be considered when counseling families with affected children.

Future efforts to identify environmental risk factors should be considered at least as important, if not more important than, finding candidate genes for ASD because environmental factors represent potential modifiable risks more amenable to prevention or intervention strategies at the population and individual levels.

8 General discussion

This section will summarize and integrated the finding from the studies presented in sections 4-7.

8.1 Summary of results

The aetiology of autism remains largely unknown. There is consistent support for substantial genetic contribution to the aetiology, but there is also evidence that non-heritable factors play a significant role. Evidence that support the role of environmental factors in the aetiology of autism is, however, not consistent, and has been passionately debated.

As one important environmental risk factor, this thesis examined the role of maternal age at birth of the offspring by characterizing potential pathways through which the risk may be operating.

A meta-analysis examined the hypothesis that older maternal age is a risk factor for autism and demonstrated that maternal age >35 years is associated with a small to moderate increase in risk for autism. There was evidence for a dose-response relationship with older maternal age associated with increasing risk.

I also examined the hypothesis that maternal age affect the risk of autism using data from the International Collaboration for Autism Registry Epidemiology (iCARE). The association between maternal age, paternal age and autism was examined in detail using advanced statistical and visualization methods. iCARE combines population-based data from 5 countries: Australia, Denmark, Finland, Israel, Norway and Sweden, allowing highly powered studies of perinatal risk factors for autism.

More than 5 million births and 30,000 ASD cases were examined. The marginal effect of paternal and maternal age were similarly associated with ASD and with the more severe form AD. The marginal paternal age risk was more pronounced for AD. The association was verified across several heterogeneous countries and health systems. There was also evidence for a risk pattern consistent with possible assortative mating. Couples with higher differences between paternal and maternal age; 'old dad – young mom' as well as 'old mom – young dad' had higher risk for ASD in the offspring.

In the earlier meta-analysis we did not find any support for an increased risk with younger maternal age. If anything, the data supported a lower relative risk with younger maternal age. Of the studies contributing with relative risk estimates for mothers < 20 only 2 showed point estimates higher than one, none of them statistically significant. 8 studies showed point estimates of relative risk less than one of which three were statistically significant. A potential explanation is differences in the adjustment for confounding. Most of the studies selected for the meta-analysis adjust 'better' for potential confounding than we had the ability to do. Examples of such confounders include socio-economic status, ethnicity and prenatal characteristics. As a comment, without further information it is however not clear if it is a good idea to include a covariate such as socio-economic economic status in the regression models. For instance, if socio-economic status is caused by maternal age and caused by diagnosis of disease or if socio-economic status is the cause of maternal age and, at the same time, there is an (unmeasured) variable confounding disease risk and socio-economic status then including a covariate for socio-economic status may instead introduce bias.

Another potential explanation for the difference between the meta-analysis and the full cohort approach in study II is the differences in categorization of the maternal age data. In the ICARE analysis we used maternal age 20-29 years as the reference. In the meta-analysis the analyzes were performed using ages 25-29 as reference category which may end up in a slightly lower relative risk. Another possible bias arises from using categories of age when the actual age distribution is different than the median in different age intervals. We do not know if the median age in the category < 20 is almost 20 or closer to 16 which may change the estimate slightly

Next I examined a potential source underlying the maternal age-autism association. Using national Swedish registers containing all births and containing information on in-vitro fertilization (IVF) I examined the possibility that the maternal age effect in autism is due to in-vitro fertilization (IVF). Overall there was no association between IVF and autism. When, in the first study ever, the range of different IVF treatments were examined, there was evidence for an association between treatments related to male infertility and intellectual disability and, for the treatment associated with the most

profound form of male infertility, with risk for autism.

Our study show that in terms of attributable risk IVF treatment can not explain the rise in autism and neurodevelopmental diseases among children and adolescents in the last 10 to 20 years. It does however show increased risk for mental retardation generally and for autism for the rare treatment used for the most profound condition of male infertility.

Lastly, while maternal age can be seen as an environmental risk factor, it can also be viewed as a factor mediating or carrying genetic risk. To better understand the balance between environmental and genetic factors I examined the importance of genetic versus environmental factors in the aetiology of ASD more generally. Using a multi-generational cohort of more than 2 million individuals born in Sweden I studied familial clustering of autism. Relative recurrence risk was estimated in twins (mono- and di-zygotic), siblings, half siblings and cousins. Heritability was then modelled using the extended family-based information.

All classes of biological relatives of probands with ASD had increased risk for ASD. Estimated heritability for ASD was 50%. Thus, although genetic factors play an important role in the aetiology of ASD, they are of substantially lower magnitude than previously estimated. 50% of the liability for ASD was related to unique environmental factors. i.e., to experiences that are different between members of the same family. Therefore, this study provides the most convincing evidence to-date for an important role environmental factors in the aetiology of ASD.

Different cases of autism may reflect differences in the underlying (genetic) aetiology and these could be of importance. **First**, families with only one effected family member (simplex families) versus families with several members effected by autism or autism-like conditions (multiplex families) can hint at differences in the underlying genetic mechanism. It has been shown that cases from simplex families on the average have a higher load of mutations occurring de-novo (single rare mutations or copy-number-variants) while members in multiplex families may share directly inherited genetic variants. In the conduct of **study IV** we included a potentially confounding covariate indicating presence or absence of a psychiatric history at the time of birth. Including this covariate did not change the estimates of relative risk, which suggests that the

genes causing psychiatric outcomes in the parents are different than the genes in the offspring.

Secondly, when genetically tested cases of autism can sometimes be shown to overlap with other diseases of a known genetic cause and with an overlap in phenotypic expression. A few examples among many of such diseases or conditions include Angelman syndrome and Prader-Willy syndrome, Fragile X syndrome , Rett's syndrome and Tuberous sclerosis. These are frequently labelled as "non-syndromic" cases, compared with cases where autism is the primary diagnosis. While technically and economically challenging only a few years ago genetic testing of such diseases are becoming practically feasible. These cases can also have a different aetiology. In the **study III**, while most likely severely under diagnosed, we searched the registers also for ICD codes associated with such diseases and found 366 of the 2,5 million children included in the study, 48 of these among the 6,959 children with autistic disorder. These proportions are not likely to have introduced any substantial bias in our results.

8.2 Strength and limitations

8.2.1 Strength

The research presented in this thesis has many strengths. We have utilized epidemiological samples with multiple advantageous properties. First, perhaps most obvious, our studies all have unparalleled sample size, in terms of number of children as well as years of follow-up. The sample size allowed us to perform statistical comparisons with high precision compared with previous studies; in particular for small sub-groups such as high maternal and paternal age combinations or male and female offspring.

However, the sample size alone is not sufficient if the sampling mechanism and underlying sampling frame do not represent a valid dataset, but this is also among our strengths. The individuals examined include complete national birth cohorts. We included all children born across multiple birth years avoid potential selection bias. Third, when following the children for clinical diagnosis of autism and other health outcomes we have used national registers containing detailed clinical diagnostic information on all individuals in the population, children as well as parents and other

relatives. Where most of the previous studies have not been able to address the full spectrum of autism diagnosis we have analysed the narrow diagnosis, AD, as well as the diagnosis containing the wider spectrum, ASD, separately. The countries contributing data to our analyses have publicly financed, publicly available, and equally accessed health systems. In the Nordic countries there is no private psychiatric care. These qualities has made it possible to follow individuals longitudinally and prospectively and with minimum of selection biases, possibly present in other studies.

Fourth, the national registers contain detailed information on individual characteristics important in our analyses. The data is usually collected for reasons independent of both the exposure variables (maternal age) and the outcome variables (diagnosis of a neurodevelopmental disorder). The information includes confounders such as birth year, attained age of the spouse, maternal and paternal psychiatric history at the offspring birth, pre- and perinatal birth characteristics and administrative data on family relations or vital statistics. This has allowed us to perform our analyses with detailed adjustment for possible confounders.

Fifth, the longitudinal nature of the data also allowed us to adjust for temporal trends and other changes over time. For instance, we have utilized data on emigration and death (of parent or child) to censor individuals which otherwise would falsely contribute with risk time to the analyses and introduce biases.

Access to the Swedish Multi Generation Register has also allowed us to perform calculation of family clustering of ASD using a bigger sample than any earlier study and with a more detailed family data than previously used.

We have utilized appropriate modern statistical methods. These include the use of survival analysis to utilize the differences in length of follow-up, splines to examine the functional form of continuous variables, robust errors to address correlations in the data, alternative estimation techniques to relax assumptions of data from certain parametric distributions and graphical techniques. Statistical methods are however not an independent component in epidemiological research but a bidirectional process: good data supports the use of advanced statistical methods, and knowledge of statistical methods is key when deciding what data to use.

For example, the properties described above, allowed us to address sex differences across all our comparisons; calculated heritability and relative recurrence risk using the most complex family structures including full siblings, maternal- and paternal half siblings and cousins defined from the same siblings; the relative recurrence risk has been described for a boy exposing boy or girl and also in the opposite direction. For the study of assisted reproduction treatment as a risk factor for autism we produced the most detailed study, up to this date, in terms of fertility treatments; longest in follow-up, most flexible in allowing and exploring the functional form and the use of informative comparison groups.

We have relied on Swedish data for two of our studies, the IVF study and the study on family risk. As a contrast to the national Swedish data, the ICARE data allowed comparison of results across a wide range of different health systems increasing the generalizability of the results.

Meta-analysis is most advantageous when there is heterogeneity across studies. It is however not always possible to utilize this feature. Here we managed to first identify confounding covariates measured on a study level and then to utilize this information in a meta regression where some of the variation between studies could be attributed to differences in the confounding covariates.

A common problem in any statistical analysis is the presence of missing data and drop-outs. However, our data are primarily register based and contain very little missing data. Where there has been drop-outs we have met this potential problem in the choice of statistical methods by censoring individuals from follow-up.

8.2.2 Limitations

The presented studies do have some limitations. The data sources we used included information on clinical ICD-10 diagnosis but were lacking the complete underlying clinical picture. We did not have any clinical assessments of traits or clinical symptoms of ASD. One such example is heritability calculations. We can not rule out measurement error problems with possible lack of specificity for the underlying liability which may bias the heritability downwards. However, this would most likely affect other studies similarly. Any diagnostic system, such as ICD, has limitations. For example,

while there are codes for autism and codes for intellectual disability there is no code that separate autism with and without intellectual disability.

We did not have access to socio-economic data which can be a possible source for confounding. Even though the register data we used come from health systems with equal access, one can not entirely rule out that there are differences in ascertainment by level of education, income and geographic areas.

A third set of limitations are the restrictions that come with non-experimental data. Where humans prefer partners and mating with partners of roughly the same age, an optimal design for the study of parental age would select partners for mating randomly. We tried to address this analytically by sub-dividing data to break dependencies and by considering the maternal and paternal age as a bivariate process.

8.3 Integration of findings and earlier research

The results and conclusions of this thesis add on to earlier research in several important ways.

Maternal age - iCARE study and meta-analysis

There has been considerable attention to the potential role of maternal age as a risk factor for ASD, but with conflicting results. We summarized and integrated this information in a systematic way. First, in a meta-analysis we combined already published studies and showed an overall statistically significantly increased risk by advancing maternal age; RR=1.3 (95% CI: 1.2-1.4) for mothers >=35 compared with mothers 25-29. While less strong and outside the primary focus of our investigations, the meta-analysis also indicated a reduced risk among younger women, RR=0.8 (95% CI: 0.6-1.0). In addition to providing strong support for a general role of maternal age in the risk of autism the study also suggested a factor possibly modifying the risk; offspring sex. Studies with higher proportion boy offspring showed slightly elevated risk by advancing maternal age. When addressing this question in the detailed analyses using the multinational ICARE cohort the modifying effect of offspring sex was nullified.

In the multinational cohort based study we verified the overall message from earlier studies. Compared with some individual studies, as often the case when combining several data sources, our analysis included less detailed data for confounding

compared with other studies (Hultman et al. 2011; Grether et al. 2009; Larsson et al. 2005). We did however manage to address issues not resolved elsewhere.

First, using splines, the detailed functional form of maternal age vs autism risk showed increased risk for all contributing sites, less pronounced in Norway and Australia, both using service registers.

Secondly, in any study looking at human parental age the maternal and paternal age will be closely related. The high power of the ICARE cohort allowed us to separate these two factors better than previously done by analysing parental age in subgroups of the spouse. This again verified the risk associated with advancing maternal age.

Analysing maternal and paternal age jointly has been done earlier by considering parental age categorically (Parner et al. 2012) There, having two parents of advanced age did not increase the risk beyond having only one parent of advanced age. For this reason they concluded that spontaneous genomic alternation is not the main mechanism through which parental age impacts ASD. We examined maternal and paternal age continuously and jointly using thin-plate splines in an approach never used before for this question. We showed that the risk of autistic offspring increases both with increasing age, highest when having both an old father and an old mother, and with increasing differences in age, in all directions, between the mother and father (**Study II, figure 3**).

Our results agree with what is expected following assortative mating (Merikangas 1982) where individuals with a similar trait (phenotype) and associated genotype mate to a higher degree than expected, or, following secondary assortative mating between the trait and another, opposite trait. In the review from 1982 Merikangas described and reviewed several studies involving effects of assortative mating on psychological traits and psychiatric illness (Merikangas 1982).

A few other examples are worth mentioning. Assortative mating has previously been shown for schizophrenia where offspring to schizophrenic parents have an increased risk of developing schizophrenia themselves (Parnas 1985) .

A recent paper, including twins, their siblings and their parents, examined the correlation between height and IQ and if the observed correlation is due to genetic

correlations from genes that affect both traits, pleiotropy, or if it can be explained by assortative mating. It concluded that both pleiotropy and assortative mating contribute significantly and about equally to the genetic correlation (Keller et al. 2013).

In a large British study increasing husband-wife height difference was associated with abnormal pregnancy outcomes (Mascie-Taylor & Boldsen 1988). This phenomena has also been observed in animal models testing the effects of mutual mate preferences on reproductive success. Female and male house mice who tested individually and mated with preferred partners had higher reproductive success and better progeny performance than individuals mated with non-preferred partners (Drickamer et al. 2003). Assortative mating have also been documented among parents of extremely obese children and adolescents (Hebebrand et al. 2000).

The observed pattern of increasing risk for autism in offspring with a higher degree of dissimilarity in parental age may also be influenced by socio-economic factors. In a US twin sample the negative correlation between paternal age and different tests scoring for IQ in children turned the effect of paternal age statistically insignificant for most developmental measures once family characteristics in general and mother's education in particular were controlled for (Edwards & Roff 2010) .

IVF treatment

Treatments of In-vitro fertilization (IVF) have increased rapidly since first introduced in 1978. IVF is associated with increasing parental age in both the mother and father. In parallel with the increase of IVF the age of parenting has increased as well (Bray et al. 2006; Kirmeyer & Hamilton 2011). Since both these factors have been associated with autism it was important to study the role of IVF in autism. While the risk associated with IVF is not limited to neurodevelopmental disorders these are usually presented at an earlier age than for instance cancer and cardiovascular diseases.

As a complement to the original IVF treatment for female fertility problems, the intra-cytoplasmic sperm injection (ICSI) was introduced in 1992 for treatment of male infertility problems. The techniques have since evolved further. A standard procedures involve freezing of embryo as an addition to the transfer of fresh embryos.

While the focus of our research has been on autism in this study we also included

intellectual disability (ID). With the overlap in phenotype and genotype between these two conditions (Betancur 2011) including ID boosted the power of the study which is especially important here. It also helps when addressing different aspects of neurodevelopment by contrasting the more social aspects associated with autism with the intellectual dimension of ID.

A possible mechanism linking IVF and neurodevelopmental disorders is epigenetic modifications (Schanen 2006; Dada et al. 2012). Epigenetic processes have been associated with Rett's (Robertson & Wolffe 2000) and Angelman's syndromes (Mann & Bartolomei 1999) , disorders characterized by autistic-like features in some cases. Experiments in mice have suggested that some of the steps involved in IVF might be related to epigenetic defects (Paoloni-Giacobino & Chaillet 2004; De Rycke et al. 2002). Mammal embryos cultured in-vitro are also susceptible to imprinting control (De Rycke et al. 2002). The risk of epigenetic changes may be modified the longer an embryo spends in culture. Although blastocyst transfer is rare and also involves sperm selection, it offers an indirect test of this hypothesis.

It is well established that that IVF treatment increases the risk for pre-term birth and congenital malformations (Källén et al. 2010) and several studies examined the role of IVF in autism and intellectual disability.

Already in 1998 the first study on ICSI and mental development was published (Bowen et al. 1998b). The study only included 89 ICSI, 84 IVF, and 80 spontaneously conceived children and followed them only up to the age of one. Using IQ scales they presented statistically significantly increased risk for delayed mental development before age one. Essentially the same study was repeated a few years later following-up the same cohort. Now, concluding no increased risk for delayed mental development, following the children up to the age of 5 (Leslie et al. 2003). A study of children born 1982-1995 in Swedish IVF clinics that used retrospective outcome data from habitation clinics following the children up to 14 years of age showed increased risk of developmental delay among all children, but not when restricting to singletons. Pinborg studied the risk for AD and ID in 3,393 twins and 5,130 singletons following IVF/ICSI treatment and 20,239 spontaneous twins born in Denmark 1995-2000 with age at follow-up 2-7 years. They did not detect any risk for ID or AD (Pinborg et al. 2004).

There are several limitations to studies done prior to the one included in this thesis. Instead of using solid epidemiological samples most studies are under-powered, using small clinically ascertained samples with a short follow-up and lack of adjustment for possible confounding factors. Instead of analysing specific treatments for infertility they are evaluating IVF generally versus spontaneously conceived children. While this is an appropriate approach from a public health perspective this is not necessarily the "correct" comparison for evaluating the risk associated with IVF. This was pointed out in study involving a random sample of children born in Great Britain 2000 to 2002, singletons and twins. This study found effect of IVF/ICSI on cognitive development compared with the general population, but not when comparing with a control group resembling the group found in infertility clinics (Carson et al. 2010).

My study examining the association between IVF treatments and AD and ID aimed to overcome the shortcomings in these earlier studies. We included the biggest sample of children following IVF treatment and controls. We used prospective data and longer follow-up than done in previous studies. We included a detailed adjustment for confounding, and, adjusted properly for differences in length of follow-up for different children. Finally, and perhaps most important, where most earlier studies compared children born following IVF and spontaneously conceived children we included detailed treatment information sub-dividing and comparing the treatments in six different groups; Standard IVF (the original IVF treatment from 1978) using fresh or frozen embryo, ICSI using fresh and frozen embryo and ICSI using fresh and frozen embryo but following surgery to extract sperms.

We found only one earlier study that compared with the one in the thesis: a cohort study of autistic spectrum disorder from 2011 that also included detailed control for confounding but only 9 years of follow-up, a sample size one-fourth of ours, and no information on specific procedures (Hvidtjørn et al. 2011).

Familial risk and heritability

First, we showed that genetic and non-genetic, environmental risk factors are equally important in ASD aetiology. We also showed that the environmental component is exclusively of non-shared origin. This is in some contrast to previous research. Autism is considered one of the most heritable of all psychiatric disorders. There is however

considerable variation among the studies investigating this. Earlier twin studies (Bailey et al. 1995; S Folstein & Rutter 1977; Steffenburg et al. 1989; Lichtenstein et al. 2010; Ronald et al. 2006), have estimated the heritability, measuring how much of the liability for autism is attributed to underlying genetic factors, to more than 90%. These first studies were based on twins only, were conspicuously small, and based on clinically based samples. The high heritability of autistic traits, close to 80%, was again shown in a population based UK twin sample of 3,500 twins (Ronald et al. 2006) . A major Swedish twin-study with more than 10,000 twins (Lichtenstein et al. 2010) reported 80% with no shared environment. In contrast, a California twin-study of showed different results with only 38% heritability and a support for considerable influences of shared environment (Hallmayer et al. 2011).

Our study on autism familial risk overcame several of the problems in these earlier studies by including more detailed family relations, bigger sample size and longer time for follow-up. The more detailed family structures utilized here allowed us to use a statistical model capable of better separating the different genetic and environmental components. Other influences on the estimates are more difficult to overcome. For instance, it can not be ruled out that twins are being diagnosed in pairs instead of independently of each other which may inflate the genetic contribution by imposing a false correlation between the twins. A factor acting in the other direction is the influence of measurement errors. A trait measured with high variation will inflate the environmental component.

For families with autistic children as well as for the purpose of family counselling there is need for more individualized measures applicable to specific family circumstances in terms of understanding familial risk. For this purpose we calculated relative risks associated with different family-member exposures. This included one relative risk having a full sibling with autism, and another relative risk having a maternal full sibling with autism. Earlier studies have mostly calculated the absolute risk for offspring autism conditioned on autism among other family members (Ritvo et al. 1989; Szatmari et al. 1998; Bolton et al. 1994; Chudley et al. 1998; Sumi et al. 2006; Constantino et al. 2010; Ozonoff et al. 2011) but this is a very complicated procedure that involves several potential obstacles. The absolute risk, i.e. the prevalence, for

autism depends on the age and birth year of the child and varies between different geographical areas which makes different recurrence risk estimates difficult to compare. The relative recurrence risk of autism was proportional to the average amount of shared genes of the family members which also supports the genetic aetiology of autism. Besides our study we only found one similar study that calculated relative recurrence risk, a study from Denmark published August 2013 (Grønborg et al. 2013) . The Danish study is using a similar data sample as in our study but a smaller sample size and less detailed family relations. The study from Denmark report RR slightly lower than we do, $RR_{full\text{-}sib}$=7.5 compared with our $RR_{full\text{-}sib}$=10.4 and $RR_{maternal\text{-}half\text{-}sib}$=2.4 compared with our $RR_{maternal\text{-}half\text{-}sib}$=3.3 and $RR_{paternal\text{-}half\text{-}sib}$=1.5 compared with our $RR_{paternal\text{-}half\text{-}sib}$=2.9. As maternal half-siblings share mother-specific exposure during pregnancy the higher risk for maternal half-siblings compared with paternal half-siblings indicates that environment exposure specific for maternal pregnancy may be of some importance.

The adjustment due to confounding were mainly explained by birth cohort effects. Additional adjustment only effected our estimates slightly. The Danish study was close to identical with and without adjustment for confounding.

How can a condition be “genetic” but not always run in the family? Psychiatric history in the family has previously been associated with an increased risk of autism (Sullivan et al. 2012; Jokiranta et al. 2013). As parental age and/or parental psychiatric history can be argued to be on a familial path related to the underlying causes of autism we fitted models both with and without these covariates included. When adjusting for parental psychiatric history the relative recurrence risk remained essentially unchanged. This suggest low penetrance or different genetic causes underlying the parental (family) psychiatric history than underlying the risk of autism in the children that control the recurrence risk under study. Yet another explanation is that many cases are caused by new mutations, mutations that arise in the germ-line of the parents; de-novo.

Our results can also be compared with the RR for schizophrenia, a neurodevelopmental disorder with earlier overlap in diagnosis and with some shared clinical and aetiological features(Stone & Iguchi 2011). In a sample overlapping with the parents and

grandparents of our study the full sibling schizophrenia RR was estimated to 8.5(Lichtenstein, Björk, et al. 2006) , 2.5 between half siblings and 2.3 between cousins. Estimates slightly lower than ours but still higher than the estimates from the Danish study.

The study of family clustering can also be approached from the perspective of the change in prevalence over time. The fast increase in autism prevalence for children born during the 1990s is not accompanied by a similar increase in relative recurrence risk. Only for children born after the year 2002 the relative recurrence risk show any increase compared with other birth cohorts (**Study IV, figure 1 & figure 2**). In other words, irrespective if the cause is genetic or comes from the environment the risk factors behind the familial risk between siblings have not changed over time. Alternatively, the changes act multiplicatively and are therefore not influencing the relative recurrence risk. This pattern was similar in the Danish study.

Potential biological mechanisms for the association between advancing maternal age and autism

One possible explanation for the maternal age effect is an increased occurrence of genomic alterations. Numerous neurological and psychiatric disorders have been related to genomic alterations (Reichenberg et al. 2009). Maternal age is an important factor in the aetiology of chromosome anomalies (Ginsburg et al. 2000; Martin 2008) and genomic modifications (Kaytor et al. 1997; Orr & Zoghbi 2007). Interestingly, a number of studies have uncovered an increased prevalence of de-novo copy-number variants (CNVs), and other forms of genomic alterations, in autistic children (Christian et al. 2008; Marshall et al. 2008; Sebat et al. 2007), supporting the notion that novel mutational events may be important in the pathogenesis of autism. Mutation can happen in the germline or somatic cells. Whether these events are also related to advancing maternal age remains to be determined.

An alternative explanation is that epigenetic dysfunction underlies some parental age effects. ‘Epigenetics’ refers to the heritable, but reversible, regulation of gene expression (Henikoff & Matzke 1997). Epigenetic dysfunction has been associated with several neuropsychiatric disorders (Mill et al. 2008), and is also implicated in single-gene disorders, including Rett's and Fragile X syndromes, characterized by autistic-like

features in some patients(Reichenberg et al. 2009).

It is possible that the accumulated exposure to various environmental toxins over the life-course could result in genomic and/or epigenetic alterations in the cells of older parents. Toxins have been shown to induce DNA damage, germline mutations and global hypermethylation (Yauk et al. 2008) in germ cells, and have long term developmental consequences in offspring (Williams & Ross 2007). In addition, increasing maternal age may be related to endocrine and hormonal factors and immunological changes, not only by ageing alone but also through maternal stress (Newschaffer et al. 2007). There is also an increase in exposure from confounding factors such as pre-term birth and pre-eclampsia with advancing maternal age (King et al. 2009; Baxter et al. 2007).

The associations between advancing maternal age and autism as well as the familial risk were consistent for AD and ASD even though some studies reported differences for the different sub-categories of the PDD classification in DSM IV (Lampi et al. 2013). It is possible that the different traits underlying the different ASD have different aetiology and different effects with maternal age. We did find differences between AD and ID following IVF treatment where we did not detect any increase in risk for AD when restricting to singleton births, but risk remained following ICSI treatments, mainly used for male-factor infertility in Sweden. This may be due to differences in power. Alternatively one can speculate, although this information does not exist as a diagnosis in the ICD-10 diagnostic system, weather the risks differ for autism with intellectual disability and autism without intellectual disability. In a Swedish study obstetric sub-optimality (prematurity, low Apgar scores, growth restriction) was positively associated with autism but not with Asperger's syndrome (Haglund & Källén 2011).

8.4 Future directions

In 2013 the DSM 5 was released. Importantly for autism the Asperger's Disorder, Atypical autism and PDD are now incorporated in the same category. When the earlier DSM IV was released it was not yet known that Rett's syndrome is caused by mutations in the MeCP2 gene. In DSM V Rett's syndrome is no longer included. There is some

support to the idea that the new DSM V will be more specific but with slightly lower sensitivity compared with the DSM IV-TR (Frazier et al. 2012; McPartland et al. 2012) The consequence is of a shift to diagnosis closer to the autistic disorder and children with early onset of the symptoms. The diagnostic change in autism thus continuous with the changes with DSM V. It is also not clear how the changes in DSM V will affect the next version of the ICD. For these reasons confounding for temporal trends will be important to consider in future studies.

There is now very strong support for the role of paternal and maternal age on ASD and neurodevelopmental disorders generally. However, improved control for family confounding and extension to three generations is needed. The three generation approach can give useful results for better understanding the role of de-novo and possible epigenetic influences. The genetic data showing overlapping genetic mechanism over a wider set of neurodevelopmental disorders and conditions such as epilepsy, intellectual disability and genetic diseases should be challenged from an epidemiological perspective and incorporated in future studies. Differences in aetiological risk factors can feed useful results back to the genetic results.

There will be a need for epidemiological studies of even higher power and complexity. However, the different countries with national registers useful for research have concerns regarding allowing possibly sensitive personal data to leave the country. Also, combining data from several sources and with complex information on family structure and diagnosis results in big data(sets). Data sharing, technical as well as multinational collaboration, will become key for achieving this.

Environmental pollutions have been suggested as triggers for ASD (Becerra et al. 2013), perhaps as a consequence of a genetic predisposition or together with epigenetic processes. This needs to be addressed but measurements are extremely difficult since data currently available is not usually on an individual exposure level but connected to roads or geographic areas. In addition, such measurements can also show big variations following yearly variations in temperature and economic activity. Exposures are most likely confounded by socio-economic status and the dose-response or concentration-response is unknown. It may be necessary to create dedicated cohorts building up exposure databases of detailed subject-specific data. This can be done

using modern techniques where measurement devices are attached to individuals and followed over time.

Another topic to explore is the geographic heterogeneity in aetiology. Even on a local or national level there may be important differences both in genetic material and in exposures. Such an approach can follow the geographic map as well as follow different economic maps where different local regions, possibly separated by long distances, can have similarities in terms of exposure, such as different exposures to immigration, pollutions and temperatures.

In our research we have relied on the use of national registers which helped us to get around several problems present in clinically based samples. While we can follow the actual diagnosis of ASD in a real population there is a lack of data connecting these observations to the underlying biology. For this reason initiatives to enrich the register data with biological data would be valuable. In some places such data is available, again, on a national level, using individual medical records and available to patients and treating doctors. Another approach is to use clever statistical designs where data can be collected in sub-cohorts. For this approach to be successful it will be important to have exact and reliable information on the underlying population from which sub-cohorts are created.

The results showing support for assortative mating of sociological influences need to be tested in new or extended populations with better control for confounding.

It has been debated how much of the increase in prevalence since the early 1990s is due to increases in exposures, if any, and how much is due to changes in the general awareness and availability of health care. This has not yet been addressed properly. For this to be addressed successfully, reliable data on socio-economic variables and income needs to be included. Such an approach could also be useful for further exploring the role of assortative mating since factors associated with mating can also be associated, or confounded, with these variables.

Follow up on the **study III** showing convincing evidence for an association between AD and ID with certain IVF treatments is needed. It is not entirely clear if the association is due to the treatments or due to the underlying infertility factors. Replication in another

population and health system is needed to verify the results. Furthermore, since the risk to a great extent seems to be associated with multiple births an interesting approach would be to analyse the "vanishing twins". Using ultra sound, twins can be detected early, but sometimes at birth there is only one child live born due to spontaneous abortion. The risk, ID or AD, following IVF treatment can be associated with vanished twins (Anand et al. 2007). Also, the highest risk was associated with IVF treatment using sperms being surgically extracted. This procedure is used for two different infertility factors, one where the tube is blocked and the sperm can not be ejaculated and the other where the sperms are of poor quality and sperms have to be retrieved from the testis directly. Separating the two procedures could be informative for understanding the mechanism underlying risk.

9 Conclusions

In conclusion, our studies have shown:

- The strongest support to this date for increasing risk of AD and ASD with advancing maternal age.
- The first large documented support for social interaction effects or assortative mating in the risk of AD and ASD.
- No support to the hypothesis that the risk of autism due to parental age is different in male or female offspring

- No support for the hypothesis that the maternal age association with risk for AD is due to IVF treatment in older women. While the original IVF treatment is safe, there is an increased risk of AD following the most severe form of male infertility using surgical extraction of the sperm. This risk is potentially modifiable by the use of single embryo transfer.

- Support for the importance of genetic factors in the aetiology of autism. The relative recurrence risk of autism is proportional to the average amount of shared genes between family members
- Strong evidence that he genetic and non-genetic factors contribute equally to the population risk of AD and ASD

10 References

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11 Appendix A - Autism diagnostic criteria

The DSM IV-TR criteria for autism include five different disorders of which each is described below.

11.1 Autistic Disorder

The DSM IV-TR criteria require:

A) Six or more items from (1), (2), and (3), with at least two from (1), and one each from (2) and (3):

   1) qualitative impairment in social interaction, as manifested by at least two of the following:

      a) marked impairment in the use of multiple non-verbal behaviours such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction

      b) failure to develop peer relationships appropriate to developmental level

      c) a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest)

      d) lack of social or emotional reciprocity

   2) qualitative impairments in communication as manifested by at least one of the following

      a) delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)

      b) in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others

      c) stereotyped and repetitive use of language or idiosyncratic language

      d) lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level

3) restricted repetitive and stereotyped patterns of behaviour, interests, and activities, as manifested by at least one of the following

   a) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus

   b) apparently inflexible adherence to specific, non-functional routines or rituals

   c) stereotyped and repetitive motor manners (e.g., hand or finger flapping or twisting, or complex whole-body movements)

   d) persistent preoccupation with parts of objects

B) Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years: (1) social interaction, (2) language as used in social communication, or (3) symbolic or imaginative play

C) The disturbance is not better accounted for by Rett's Disorder or Childhood Disintegrative Disorder

11.2 Asperger's Disorder

A) qualitative impairment in social interaction, as manifested by at least two of the following:

   1) marked impairment in the use of multiple non-verbal behaviours such as eye-to eye gaze, facial expression, body postures, and gestures to regulate social interaction

   2) failure to develop peer relationships appropriate to developmental level

   3) a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest to other people)

   4) lack of social or emotional reciprocity

B) Restricted repetitive and stereotyped patterns of behaviour, interests and activities, as manifested by at least one of the following:

   1) encompassing preoccupation with one or more stereotyped and restricted

patterns of interest that is abnormal either in intensity of focus

2) apparently inflexible adherence to specific, non-functional routines or rituals

3) stereotyped and repetitive motor mannerisms (e.g., hand or finger flapping or twisting, or complex whole-body movements)

4) persistent preoccupation with parts of objects

C) The disturbance causes clinically significant impairment in social, occupational, or other important areas of functioning.

D) There is no clinically significant general delay in language (e.g., single words used by age 2 years, communicative phrases used by age 3 years).

E) There is no clinically significant delay in cognitive development or in the development of age-appropriate self-help skills, adaptive behaviour (other than in social interaction), and curiosity about the environment in childhood.

F) Criteria are not met for another specific Pervasive Developmental Disorder or Schizophrenia

11.3 Pervasive Developmental Disorder Not Otherwise Specified

This category should be used when there is a severe and pervasive impairment in the development of reciprocal social interaction associated with impairment in either verbal or non-verbal communication skills or with the presence of stereotyped behaviour, interests, and activities, but the criteria are not met for a specific Pervasive Developmental Disorder, Schizophrenia, Schizotypal Personality Disorder, or Avoidant Personality Disorder. For example, this category includes "atypical autism" - presentations that do not meet the criteria for Autistic Disorder because of late age at onset, atypical symptomatology, or sub-threshold symptomatology, or all of these.

11.4 Rett's Disorder

A) All of the following:

1) apparently normal prenatal and perinatal development

2) apparently normal psycho motor development through the first 5 months after

birth

3) normal head circumference at birth

B) Onset of all of the following after the period of normal development:

1) deceleration of head growth between ages 5 and 48 months

2) loss of previously acquired purposeful hand skills between 5 and 30 months with the subsequent development of stereotyped hand movements (e.g., hand-wringing or hand washing)

3) loss of social engagement early in the course (although often social interaction develops later)

4) appearance of poorly coordinated gait or trunk movements

5) severely impaired expressive and receptive language development with severe psycho motor retardation

11.5 Childhood Disintegrative Disorder

A) Apparently **normal development for at least the first 2 years** after birth as manifested by the presence of age-appropriate verbal and non-verbal communication, social relationships, play, and adaptive behaviour

B) Clinically significant loss of **previously** acquired skills (before age 10 years) in at least two of the following areas:

1) expressive or receptive language

2) social skills or adaptive behaviour

3) bowel or bladder control

4) play

5) motor skills

C) Abnormalities of functioning in at least two of the following areas:

1) qualitative impairment in social interaction (e.g., impairment in non-verbal behaviours, failure to develop peer relationships, lack of social or emotional reciprocity)

2) qualitative impairments in communication (e.g., delay or lack of spoken language, inability to initiate or sustain a conversation, stereotyped and repetitive use of language, lack of varied make-believe play)

3) restricted, repetitive, and stereotyped patterns of behaviour, interest, and activities, including motor stereotypes and mannerisms

D) The disturbance is not better accounted for by another specific Pervasive Developmental Disorder or by Schizophrenia\

12 Appendix B - Study III Online Supplementary Material

The tables presented here were published as supplementary online tables in the JAMA manuscript.

eTable 1 Description of Swedish population based registers

eTable 2 ICD codes for autistic disorder, mental retardation, genetic diseases and parental psychiatric history

eTable 3 Autistic Disorder. Comparing Any IVF vs Spontaneously conceived children. Relative risk (RR) and two-sided 95% confidence intervals (CI). RR presented for crude models (adjusting for age, sex and birth year only) and adjusted models (additionally adjusting for confounding). RR presented also for the supplementary analyses adjusting for calendar time (AdjC), adjusting for years of infertility (Adj I) and for subgroups of male and female children; subgroups of pre-term and term born children; subgroup of children born after 1998. All calculations presented for multiple birth and singletons separately. Supplementary results in italic.

eTable 4 Mental Retardation. Comparing Any IVF vs Spontaneously conceived children. Relative risk (RR) and two-sided 95% confidence intervals (CI). RR presented for crude models (adjusting for age, sex and birth year only) and adjusted models (additionally adjusting for confounding). RR presented also for the supplementary analyses adjusting for calendar time (AdjC), adjusting for years of infertility (Adj I) and for subgroups of male and female children; subgroups of pre-term and term born children; subgroup of children born after 1998. All calculations presented for multiple birth and singletons separately. Supplementary results in italic.

eTable 5 Autistic disorder. Comparing specific IVF procedures vs IVF without ICSI, fresh embryo. Relative risk (RR) and two-sided 95% confidence intervals (CI). RR presented for crude models (adjusting for age, sex and birth year only) and adjusted models (additionally adjusting for confounding). RR presented also for the supplementary analyses adjusting for calendar time

(AdjC), adjusting for years of infertility (Adj I) and adjusting for diagnosis of genetic disease (Adj G) and for subgroups of male and female children; subgroups of pre-term and term born children; subgroup of children born after 1998. All calculations presented for multiple birth and singletons separately. Supplementary results in italic.

eTable 6 Mental Retardation. Comparing specific IVF procedures vs IVF without ICSI, fresh embryo. Relative risk (RR) and two-sided 95% confidence intervals (CI). RR presented for crude models (adjusting for age, sex and birth year only) and adjusted models (additionally adjusting for confounding). RR presented also for the supplementary analyses adjusting for calendar time (AdjC), adjusting for years of infertility (Adj I) and adjusting for diagnosis of genetic disease (Adj G) and for subgroups of male and female children; subgroups of pre-term and term born children; subgroup of children born after 1998. All calculations presented for multiple birth and singletons separately. Supplementary results in italic.

eTable 7 Autistic disorder. Comparing IVF techniques. Each "technique" defined by combining specific IVF procedures sharing the same technique. Relative risk (RR) and two-sided 95% confidence intervals (CI). RR presented for crude models (adjusting for age, sex and birth year only) and adjusted models (additionally adjusting for confounding). RR presented also for the supplementary analyses adjusting for calendar time (AdjC), adjusting for years of infertility (Adj I) and adjusting for diagnosis of genetic disease (Adj G) and for subgroups of male and female children; subgroups of preterm and term born children; subgroup of children born after 1998. All calculations presented for multiple birth and singletons separately. Supplementary results in italic.

eTable 8 Mental Retardation. Comparing IVF techniques. Each "technique" defined by combining specific IVF procedures sharing the same technique. Relative risk (RR) and two-sided 95% confidence intervals (CI). RR presented for crude models (adjusting for age, sex and birth year only) and adjusted models (additionally adjusting for confounding). RR presented also for the

supplementary analyses adjusting for calendar time (AdjC), adjusting for years of infertility (Adj I) and adjusting for diagnosis of genetic disease (Adj G) and for subgroups of male and female children; subgroups of pre-term and term born children; subgroup of children born after 1998. All calculations presented for multiple birth and singletons separately. Supplementary results in italic.

eTable 9 Hormones. Comparing children born following hormone treatment as only fertility treatment vs children spontaneous conceived without use of hormones. Relative risk (RR) and two-sided 95% confidence intervals (CI) for autistic disorder and for mental retardation for. For All children and for singletons. All RR from adjusted models.

eTable 10 Comparisons of children born following specific IVF procedures vs children born after spontaneous conception. Relative risk (RR) and two-sided 95% confidence intervals (CI) for autistic disorder and for mental retardation for. For All children and for singletons. All RR from adjusted models.

eTable 11 Comparing the risk for autistic disorder and mental retardation in multiples vs singletons. Results are presented separately for children following any IVF treatment and spontaneous conception. Multiples include any birth with > 1 live born child. Singletons include birth with one live born child.

eTable 12 All children. Distribution of confounders and children characteristics for spontaneous conceived with and without hormone treatment. Hormone treatment being the only treatment for fertility.

eTable 1. Description of Swedish population based registers

Register	Description
Swedish Medical Birth Register#	The Medical Birth Register was established in 1973. It contains data on pregnancy and birth for all births in Sweden. More than 95% of the Swedish pregnant population attend antenatal care before the 15th gestational week and the register covers over 99% of all births. This register includes information collected prospectively, starting with the first antenatal visit through the time when mother and child are discharged from the hospital after delivery. Antenatal care routines are standardized and the information is provided through antenatal, obstetrical, and neonatal records, and classified according to the International Classification of Diseases (ICD) version 8 until 1986, version 9 from 1987 to 1996, and version 10 subsequently. Virtually all pregnant women attend an antenatal clinic. During the first visit, usually during pregnancy week 8-13, the woman is asked about the number of years of involuntary infertility. The information is recorded in the Medical Birth Register.
Swedish IVF registers#	Frequencies of all IVF/ICSI treatment in Sweden from 1982 to 2007. From 2007 data are stored in a separate Swedish "quality register". Since 2003 data on embryos transferred are registered as well. The 16 clinics for IVF/ICSI Sweden are required by law to report all treatments. IVF/ICSI treatments are offered to women in the range 25-42 years of age. There are no strict age restrictions for males. Eligibility requires a medically documented fertility problem. In Sweden, almost exclusively, IVF is used to treat female infertility while ICSI is used for male infertility. For IVF without ICSI, sperm is introduced to the egg in a dish or in a test-tube where fertilization takes place, in vitro, usually within 24 hours. The fertilized egg develops into an embryo, which is further cultivated for a total of 2-3 days, to the “cleavage stage”, or for 5-6 days

	to a ”blastocyst”. One or, occasionally, two (in previous years even more than two) embryos are then transferred to the uterus in a “fresh embryo transfer”. Excess embryos can be frozen and later thawed for a second, now frozen-and-thawed, embryo transfer. When ICSI treatments are applied, one single sperm is injected directly into the cytoplasm of the egg where fertilization later takes place. For more serious cases of male infertility when no, or very few, sperm are found in the ejaculate they can be surgically extracted from either the testis or the epididymitis and used in the ICSI procedure.
Multi Generation Register	The Multi Generation Register contains information about the entire Swedish population. Children born from 1932 and alive 1961 are linked to their biological parents. The register comprises 9 million children (index persons), and 11 million unique individuals. Importantly the register includes family information (e.g., identification of parents, siblings and offspring)allowing linkage to other population based registers, which include information on health (e.g. psychiatric hospitalizations), demographic variables (e.g., date of birth, death and emigration).
National Patient Register#	Sweden has universal and publicly financed health insurance coverage that guarantees equal access to health services, regardless of employment status, socio-economic status or regional residency. The register has a nationwide coverage of patient treatment facilities and includes care in psychiatric as well as somatic hospitals. There are no private psychiatric hospitals in Sweden. The Swedish National Patient Register contains details on virtually all psychiatric hospitalizations since 1973. Before 1973 there is data for selected counties only. The register include data on admission and discharge dates and the discharge diagnosis made by the treating physician. Outpatient visits are included since 1999. Diagnostic information is coded using the ICD codes. The standard procedure dictates that diagnosis will be given by a consultant (equivalent of an attending) psychiatrist at the time of

	discharge from hospital. The diagnostic assessment is then forwarded on a computer medium to the National Patient Register. These routines are standardized across Sweden. All infants and preschool children are regularly seen at well-child care clinics and undergo routine medical and developmental screening. All children aged 4 undergo routine general health screening, that includes mandatory developmental assessment (motor, language, cognitive and social development) conducted by a nurse and paediatrician. Children with any suspected developmental disorder (including autistic disorder and mental retardation) are referred for further assessment by a specialized team in a child psychiatry unit or habilitation service. During the study period diagnoses were made by diagnostic teams with a psychiatrist, clinical psychologist, and speech pathologist or occupational therapist, depending on clinical manifestations. The instruments include parental interviews, cognitive testing of the child, and observations in naturalistic settings, including the home or the unit. The Patient Register contains the diagnostic information. The Patient-Register has shown high reliability for somatic and psychiatric diagnoses. Also, for 130 cases of autistic disorder, we earlier compared registry diagnosis to diagnosis according to the Diagnostic and Statistical Manual 4th Edition (DSM-IV) confirming reliability (methods and results are available from the authors on request). For a diagnosis of mental retardation the evaluation is made by a psychologist and according to standardized tests with high reliability.
Statistics Sweden Vital Statistics	Individual vital statistics data including date of birth, emigration, immigration and death is maintained by Statistics Sweden (Total Population Register, Emigration and immigration register).

#: Register owned and monitored by the Swedish National Board of Health and Welfare. More detailed documentations at www.socialstyrelsen.se/en/

Note: Data from all registers are joined by the register owner(s)by the unique Swedish personal identification number.

eTable 2. ICD codes for autistic disorder, mental retardation, genetic diseases and parental psychiatric history

Variable	Disorder	ICD codes
Severe development disorder	Autistic disorder #	ICD-9: 299/299B/W/X ICD-10: F84.0
	Mental retardation	ICD-9: 317, 318 or 319 including all sub-codes or 318A, 318B or 318C ICD-10: F70-F73, F78, F79 including all sub-codes
Parental psychiatric history	Affective disorder (mood disorder)	ICD-7: 301 and 302 ICD-8: 296, 298 and 3004.1 ICD-9: 296, 311, 298A, 298B, 300E ICD-10: F30-F34, F38, F39
	Non affective psychosis	ICD-7: 300 or 309 ICD-8: 295, 297, 299, 298.2, 298.3, 298.9 ICD-9: 295, 297, 298, 298C, 298E, 298W, 298X ICD-10: F20-F25, F28, F29, F230-F233, F238, F239
Genetic disease##		**Fragile-X** ICD-9 759,83 or ICD19 Q992, **Angelman** ICD-9 759,89 or ICD-10 Q935, **Prader-Willi** ICD-9 759,81 or ICD-10 Q871, **Zellweger** ICD-9 277,86 or ICD-10 Q878, **William** ICD-9 758,9 or ICD-10 Q938, **Tuberous Sclerosis** ICD-9 259,5 or ICD-10 Q851, **Tourette** ICD-9 307,23 or ICD-10 F952, **Neuofibromatosis** ICD-9 237,7 or ICD-10 Q850, **Duchennes muscular dystrophy** ICD-9 359,1 or ICD-10 QG710, **Cornelia de Lange** ICD-9 759,89 or ICD-10 Q871, **DeGeorge** ICD-9 279,11 or 758,32 or ICD-10 Q821, **Smith-Lemli-Opitz** ICD-9 759.84 or ICD-10 Q871, **Klinfelter** ICD-9 758,7 or ICD-10 Q980, Q981, Q982, Q983 or Q984. There is an overlap where ICD-10 Q871 can be both de Lange and Smith-Lemli-Opitz.

#: Codes used from 1987, ##: Source: Hollander E, Kolevzon A, Coyle JT. *Textbook of Autism Spectrum Disorders*. American Psychiatric Pub; 2010.

eTable 3 Autistic Disorder. Comparing Any IVF vs Spontaneously conceived children.

Dataset	Sub-Group	Spontaneous or Any IVF	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children		Spontaneous	6,856	33,994,678	15.6		reference group	
All Children		Any IVF	103	231,118	19.0	Crude	1.22 (1.01-1.49)	0.04
All Children						Adj	1.14 (0.94-1.39)	0.18
All Children						*AdjC*	*1.22 (1.01-1.49)*	*0.04*
All Children						*AdjI*	*1.15 (0.93-1.43)*	*0.19*
All Children	*>1998*	*Spontaneous*	*2,123*	*4,708,440*	*36.0*		*reference group*	
All Children	*>1998*	*Any IVF*	*68*	*112,209*	*48.4*	*Adj*	*1.25 (0.98-1.60)*	*0.07*
All Children	*Boys*	*Spontaneous*	*5,067*	*17,453,631*	*25.3*		*reference group*	
All Children	*Boys*	*Any IVF*	*76*	*119,364*	*29.7*	*Adj*	*1.14 (0.91-1.43)*	*0.26*
All Children	*Girls*	*Spontaneous*	*1,789*	*16,541,047*	*9.6*		*reference group*	
All Children	*Girls*	*Any IVF*	*27*	*111,754*	*13.1*	*Adj*	*1.16 (0.79-1.69)*	*0.45*
All Children	*Pre-Term*	*Spontaneous*	*665*	*1,959,075*	*25.6*		*reference group*	
All Children	*Pre-Term*	*Any IVF*	*35*	*53,629*	*29.4*	*Adj*	*1.10 (0.78-1.54)*	*0.59*
All Children	*Term*	*Spontaneous*	*6,191*	*32,035,603*	*15.1*		*reference group*	
All Children	*Term*	*Any IVF*	*68*	*177,489*	*16.2*	*Adj*	*1.00 (0.79-1.28)*	*0.97*

Continues on next page

eTable 3 (cont.)

Dataset	Sub-Group	Spontaneous or Any IVF	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons		Spontaneous	6,683	33,285,383	15.0		reference group	
Singletons		Any IVF	54	149,932	14.4	Crude	0.96 (0.74-1.26)	0.78
Singletons						Adj	0.89 (0.68-1.17)	0.41
Singletons						*AdjC*	*0.96 (0.73-1.26)*	*0.77*
Singletons						*AdjI*	*0.89 (0.67-1.18)*	*0.42*
Singletons	*>1998*	*Spontaneous*	*2,068*	*4,599,526*	*36.0*		*reference group*	
Singletons	*>1998*	*Any IVF*	*36*	*79,704*	*36.1*	*Adj*	*0.92 (0.66-1.29)*	*0.64*
Singletons	*Boys*	*Spontaneous*	*4,939*	*17,095,982*	*24.3*		*reference group*	
Singletons	*Boys*	*Any IVF*	*42*	*77,261*	*23.7*	*Adj*	*0.94 (0.69-1.27)*	*0.68*
Singletons	*Girls*	*Spontaneous*	*1,744*	*16,189,401*	*9.1*		*reference group*	
Singletons	*Girls*	*Any IVF*	*12*	*72,671*	*8.4*	*Adj*	*0.77 (0.43-1.35)*	*0.36*
Singletons	*Pre-Term*	*Spontaneous*	*596*	*1,663,935*	*25.0*		*reference group*	
Singletons	*Pre-Term*	*Any IVF*	*7*	*14,139*	*19.0*	*Adj*	*0.71 (0.34-1.50)*	*0.37*
Singletons	*Term*	*Spontaneous*	*6,087*	*31,621,448*	*14.7*		*reference group*	
Singletons	*Term*	*Any IVF*	*47*	*135,793*	*14.1*	*Adj*	*0.89 (0.67-1.19)*	*0.45*

Crude model denotes model adjusting for birth year, age and sex only; Adj model denotes adjusting for birth year, age and sex and additionally adjusting for paternal psychiatric history, maternal psychiatric history and paternal and maternal age; AdjC model denotes adjusting for birth year, age and sex, and additionally for calendar time; AdjI model denotes adjusting for birth year, age and sex, paternal psychiatric history, maternal psychiatric history and paternal and maternal age, and additionally for years of infertility. Note: x indicate cells not estimable since too few cases.

eTable 4 Mental Retardation. Comparing Any IVF vs Spontaneously conceived children.

Dataset	Sub-Group	Spontaneous or Any IVF	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children		Spontaneous	15,647	33,947,960	39.8	reference group		
All Children		Any IVF	180	230,710	46.3	Crude	1.16 (1.00-1.35)	0.04
All Children						Adj	1.18 (1.01-1.36)	0.03
All Children						*AdjC*	*1.16 (1.00-1.35)*	*0.05*
All Children						*AdjI*	*1.13 (0.96-1.32)*	*0.14*
All Children	*>1998*	*Spontaneous*	*3,408*	*4,703,484*	*70.3*	*reference group*		
All Children	*>1998*	*Any IVF*	*98*	*112,101*	*85.5*	*Adj*	*1.20 (0.98-1.47)*	*0.08*
All Children	*Boys*	*Spontaneous*	*9,429*	*17,430,939*	*47.5*	*reference group*		
All Children	*Boys*	*Any IVF*	*123*	*119,121*	*60.8*	*Adj*	*1.33 (1.11-1.59)*	*<.01*
All Children	*Girls*	*Spontaneous*	*6,218*	*16,517,022*	*33.3*	*reference group*		
All Children	*Girls*	*Any IVF*	*57*	*111,589*	*32.4*	*Adj*	*0.94 (0.73-1.22)*	*0.66*
All Children	*Pre-Term*	*Spontaneous*	*2,127*	*1,950,931*	*96.7*	*reference group*		
All Children	*Pre-Term*	*Any IVF*	*70*	*53,427*	*79.2*	*Adj*	*0.87 (0.69-1.11)*	*0.27*
All Children	*Term*	*Spontaneous*	*13,520*	*31,997,029*	*36.4*	*reference group*		
All Children	*Term*	*Any IVF*	*110*	*177,283*	*36.9*	*Adj*	*1.01 (0.84-1.22)*	*0.90*

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eTable 4 (cont.)

Dataset	Sub-Group	Spontaneous or Any IVF	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons		Spontaneous	15,178	33,240,234	38.5	reference group		
Singletons		Any IVF	101	149,677	38.8	Crude	1.01 (0.83-1.23)	0.93
Singletons						Adj	1.01 (0.83-1.24)	0.89
Singletons						*AdjC*	*1.01 (0.83-1.23)*	*0.94*
Singletons						*AdjI*	*0.95 (0.78-1.17)*	*0.64*
Singletons	*>1998*	*Spontaneous*	*3,306*	*4,594,778*	*69.8*	*reference group*		
Singletons	*>1998*	*Any IVF*	*60*	*79,605*	*74.0*	*Adj*	*1.03 (0.80-1.33)*	*0.81*
Singletons	*Boys*	*Spontaneous*	*9,136*	*17,074,246*	*45.8*	*reference group*		
Singletons	*Boys*	*Any IVF*	*68*	*77,117*	*50.1*	*Adj*	*1.13 (0.89-1.44)*	*0.31*
Singletons	*Girls*	*Spontaneous*	*6,042*	*16,165,988*	*32.3*	*reference group*		
Singletons	*Girls*	*Any IVF*	*33*	*72,561*	*28.1*	*Adj*	*0.84 (0.59-1.18)*	*0.31*
Singletons	*Pre-Term*	*Spontaneous*	*1,875*	*1,656,758*	*95.8*	*reference group*		
Singletons	*Pre-Term*	*Any IVF*	*17*	*14,068*	*66.2*	*Adj*	*0.74 (0.46-1.20)*	*0.22*
Singletons	*Term*	*Spontaneous*	*13,303*	*31,583,476*	*35.8*	*reference group*		
Singletons	*Term*	*Any IVF*	*84*	*135,610*	*36.2*	*Adj*	*1.01 (0.81-1.25)*	*0.94*

Crude model denotes model adjusting for birth year, age and sex only; Adj model denotes adjusting for birth year, age and sex and additionally adjusting for paternal psychiatric history, maternal psychiatric history and paternal and maternal age; AdjC model denotes adjusting for birth year, age and sex, and additionally for calendar time; AdjI model denotes adjusting for birth year, age and sex, paternal psychiatric history, maternal psychiatric history and paternal and maternal age, and additionally for years of infertility. Note: x indicate cells not estimable since too few cases.

eTable 5 Autistic disorder. Comparing specific IVF procedures vs IVF without ICSI, fresh embryo.

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children		IVF without ICSI, fresh	53	144,207	29.3		reference group	
All Children		IVF without ICSI, frozen	10	17,121	42.3	Crude	1.44 (0.73-2.85)	0.29
All Children						Adj	1.46 (0.74-2.89)	0.27
All Children						*AdjC*	*1.45 (0.74-2.87)*	*0.28*
All Children						*AdjI*	*1.46 (0.74-2.89)*	*0.27*
All Children						*AdjG*	*1.46 (0.74-2.89)*	*0.27*
All Children		ICSI, fresh	31	58,262	34.0	Crude	1.16 (0.73-1.85)	0.52
All Children						Adj	1.20 (0.75-1.91)	0.45
All Children						*AdjC*	*1.15 (0.73-1.83)*	*0.55*
All Children						*AdjI*	*1.19 (0.75-1.90)*	*0.46*
All Children						*AdjG*	*1.20 (0.75-1.91)*	*0.45*
All Children		ICSI, frozen	1	7,022	9.4	Crude	0.32 (0.04-2.34)	0.26
All Children						Adj	0.33 (0.05-2.40)	0.27
All Children						*AdjC*	*0.32 (0.04-2.33)*	*0.26*
All Children						*AdjI*	*0.33 (0.05-2.40)*	*0.27*
All Children						*AdjG*	*0.33 (0.05-2.40)*	*0.27*

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eTable 5 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children		ICSI, fresh, surgery	8	3,720	135.7	Crude	4.64 (2.17-9.92)	<.01
All Children						Adj	4.60 (2.14-9.88)	<.01
All Children						*AdjC*	*4.57 (2.14-9.77)*	*<.01*
All Children						*AdjI*	*4.60 (2.14-9.89)*	*<.01*
All Children						*AdjG*	*4.60 (2.14-9.88)*	*<.01*
All Children		ICSI, frozen, surgery	0	787	0.0	Crude	x	x
All Children						Adj	x	x
All Children						*AdjC*	*x*	*x*
All Children						*AdjI*	*x*	*x*
All Children						*AdjG*	*x*	*x*
All Children	*Pre-Term*	*IVF without ICSI, fresh*	*17*	*36,927*	*38.4*	*reference group*		
All Children	*Pre-Term*	*IVF without ICSI, frozen*	*3*	*3,297*	*67.9*	*Adj*	*1.69 (0.49-5.79)*	*0.40*
All Children	*Pre-Term*	*ICSI, fresh*	*10*	*11,508*	*51.6*	*Adj*	*1.47 (0.66-3.26)*	*0.35*
All Children	*Pre-Term*	*ICSI, frozen*	*0*	*1,055*	*0.0*	*Adj*	*x*	*x*
All Children	*Pre-Term*	*ICSI, fresh, surgery*	*5*	*764*	*364.5*	*Adj*	*9.54 (3.43-26.57)*	*<.01*
All Children	*Pre-Term*	*ICSI, frozen, surgery*	*0*	*78*	*0.0*	*Adj*	*x*	*x*

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eTable 5 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*Term*	*IVF without ICSI, fresh*	*36*	*107,280*	*26.6*		*reference group*	
All Children	*Term*	*IVF, without ICSI, frozen*	*7*	*13,824*	*36.4*	*Adj*	*1.39 (0.62-3.14)*	*0.42*
All Children	*Term*	*ICSI, fresh*	*21*	*46,753*	*29.0*	*Adj*	*1.11 (0.64-1.93)*	*0.72*
All Children	*Term*	*ICSI, frozen*	*1*	*5,967*	*10.9*	*Adj*	*0.42 (0.06-3.09)*	*0.40*
All Children	*Term*	*ICSI, fresh, surgery*	*3*	*2,957*	*65.0*	*Adj*	*2.42 (0.74-7.97)*	*0.14*
All Children	*Term*	*ICSI, frozen, surgery*	*0*	*708*	*0.0*	*Adj*	*x*	*x*
All Children	*Boys*	*IVF without ICSI, fresh*	*42*	*76,420*	*48.4*		*reference group*	
All Children	*Boys*	*IVF without ICSI, frozen*	*6*	*8,788*	*52.6*	*Adj*	*1.13 (0.48-2.68)*	*0.77*
All Children	*Boys*	*ICSI, fresh*	*22*	*28,635*	*49.5*	*Adj*	*1.11 (0.65-1.90)*	*0.69*
All Children	*Boys*	*ICSI, frozen*	*1*	*3,271*	*19.8*	*Adj*	*0.44 (0.06-3.24)*	*0.42*
All Children	*Boys*	*ICSI, fresh, surgery*	*5*	*1,806*	*174.0*	*Adj*	*3.80 (1.48-9.79)*	*<.01*
All Children	*Boys*	*ICSI, frozen, surgery*	*0*	*445*	*0.0*	*Adj*	*x*	*x*
All Children	*Girls*	*IVF, without ICSI, fresh*	*11*	*67,787*	*15.6*		*reference group*	
All Children	*Girls*	*IVF without ICSI, frozen*	*4*	*8,333*	*44.9*	*Adj*	*2.63 (0.84-8.29)*	*0.10*

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eTable 5 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*Girls*	*ICSI, fresh*	*9*	*29,627*	*26.7*	*Adj*	*1.50 (0.61-3.66)*	*0.37*
All Children	*Girls*	*ICSI, frozen*	*0*	*3,751*	*0.0*	*Adj*	*x*	*x*
All Children	*Girls*	*ICSI, fresh, surgery*	*3*	*1,915*	*137.7*	*Adj*	*7.31 (2.01-26.59)*	*<.01*
All Children	*Girls*	*ICSI, frozen, surgery*	*0*	*342*	*0.0*	*Adj*	*x*	*x*
All Children	*>1998*	*IVF without ICSI, fresh*	*30*	*52,992*	*43.0*		*reference group*	
All Children	*>1998*	*IVF without ICSI, frozen*	*5*	*8,131*	*55.0*	*Adj*	*1.32 (0.51-3.42)*	*0.57*
All Children	*>1998*	*ICSI, fresh*	*25*	*41,682*	*47.0*	*Adj*	*1.12 (0.65-1.90)*	*0.69*
All Children	*>1998*	*ICSI, frozen*	*1*	*5,637*	*15.9*	*Adj*	*0.38 (0.05-2.80)*	*0.34*
All Children	*>1998*	*ICSI, fresh, surgery*	*7*	*3,127*	*174.9*	*Adj*	*4.16 (1.81-9.55)*	*<.01*
All Children	*>1998*	*ICSI, frozen, surgery*	*0*	*639*	*0.0*	*Adj*	*x*	*x*
Singletons		IVF without ICSI, fresh	30	89,038	23.9		reference group	
Singletons		IVF without ICSI, frozen	5	12,309	25.9	Crude	1.09 (0.42-2.82)	0.86
Singletons						Adj	1.14 (0.44-2.95)	0.79
Singletons						*AdjC*	*1.10 (0.42-2.84)*	*0.85*
Singletons						*AdjI*	*1.13 (0.44-2.94)*	*0.80*
Singletons						*AdjG*	*1.14 (0.44-2.95)*	*0.79*

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eTable 5 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons		ICSI, fresh	18	39,931	26.0	Crude	1.09 (0.60-2.00)	0.78
Singletons						Adj	1.17 (0.63-2.15)	0.62
Singletons						*AdjC*	*1.07 (0.59-1.97)*	*0.82*
Singletons						*AdjI*	*1.15 (0.63-2.13)*	*0.65*
Singletons						*AdjG*	*1.17 (0.63-2.15)*	*0.62*
Singletons		ICSI, frozen	0	5,519	0.0	Crude	x	x
Singletons						Adj	x	x
Singletons						*AdjC*	*x*	*x*
Singletons						*AdjI*	*x*	*x*
Singletons						*AdjG*	*x*	*x*
Singletons		ICSI, fresh, surgery	1	2,569	21.9	Crude	0.92 (0.12-6.79)	0.93
Singletons						Adj	0.95 (0.13-7.09)	0.96
Singletons						*AdjC*	*0.90 (0.12-6.62)*	*0.91*
Singletons						*AdjI*	*0.94 (0.13-6.98)*	*0.95*
Singletons						*AdjG*	*0.95 (0.13-7.09)*	*0.96*

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eTable 5 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons		ICSI, frozen, surgery	0	566	0.0	Crude	x	x
Singletons						Adj	x	x
Singletons						*AdjC*	*x*	*x*
Singletons						*AdjI*	*x*	*x*
Singletons						*AdjG*	*x*	*x*
Singletons	*Pre-Term*	*IVF without ICSI, fresh*	*6*	*9,105*	*35.9*	*reference group*		
Singletons	*Pre-Term*	*IVF without ICSI, frozen*	*0*	*944*	*0.0*	*Adj*	*x*	*x*
Singletons	*Pre-Term*	*ICSI, fresh*	*1*	*3,400*	*14.0*	*Adj*	*0.37 (0.04-3.11)*	*0.36*
Singletons	*Pre-Term*	*ICSI, frozen*	*0*	*443*	*0.0*	*Adj*	*x*	*x*
Singletons	*Pre-Term*	*ICSI, fresh, surgery*	*0*	*215*	*0.0*	*Adj*	*x*	*x*
Singletons	*Pre-Term*	*ICSI, frozen, surgery*	*0*	*30*	*0.0*	*Adj*	*x*	*x*

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eTable 5 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model		
Singletons	*Term*	*IVF without ICSI, fresh*	*24*	*79,932*	*19.9*	*reference group*		
Singletons	*Term*	*IVF without ICSI, frozen*	*5*	*11,365*	*26.3*	*Adj*	*1.41 (0.53-3.71)*	*0.49*
Singletons	*Term*	*ICSI, fresh*	*17*	*36,531*	*24.2*	*Adj*	*1.35 (0.71-2.57)*	*0.37*
Singletons	*Term*	*ICSI, frozen*	*0*	*5,076*	*0.0*	*Adj*	*x*	*x*
Singletons	*Term*	*ICSI, fresh, surgery*	*1*	*2,353*	*21.5*	*Adj*	*1.19 (0.16-8.89)*	*0.87*
Singletons	*Term*	*ICSI, frozen, surgery*	*0*	*535*	*0.0*	*Adj*	*x*	*x*
Singletons	*Boys*	*IVF without ICSI, fresh*	*25*	*47,109*	*47.8*	*reference group*		
Singletons	*Boys*	*IVF without ICSI, frozen*	*3*	*6,365*	*37.1*	*Adj*	*0.84 (0.25-2.80)*	*0.78*
Singletons	*Boys*	*ICSI, fresh*	*13*	*19,618*	*46.6*	*Adj*	*1.05 (0.53-2.11)*	*0.88*
Singletons	*Boys*	*ICSI, frozen*	*0*	*2,583*	*0.0*	*Adj*	*x*	*x*
Singletons	*Boys*	*ICSI, fresh, surgery*	*1*	*1,275*	*53.7*	*Adj*	*1.16 (0.16-8.72)*	*0.88*
Singletons	*Boys*	*ICSI, frozen, surgery*	*0*	*310*	*0.0*	*Adj*	*x*	*x*

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eTable 5 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons	*Girls*	*IVF without ICSI, fresh*	*5*	*41,928*	*6.0*		*reference group*	
Singletons	*Girls*	*IVF without ICSI, frozen*	*2*	*5,944*	*16.1*	*Adj*	*2.46 (0.48-12.71)*	*0.28*
Singletons	*Girls*	*ICSI, fresh*	*5*	*20,313*	*9.2*	*Adj*	*1.67 (0.48-5.82)*	*0.42*
Singletons	*Girls*	*ICSI, frozen*	*0*	*2,936*	*0.0*	*Adj*	*x*	*x*
Singletons	*Girls*	*ICSI, fresh, surgery*	*0*	*1,294*	*0.0*	*Adj*	*x*	*x*
Singletons	*Girls*	*ICSI, frozen, surgery*	*0*	*256*	*0.0*	*Adj*	*x*	*x*
Singletons	*>1998*	*IVF without ICSI, fresh*	*19*	*36,536*	*37.5*		*reference group*	
Singletons	*>1998*	*IVF without ICSI, frozen*	*3*	*6,413*	*37.3*	*Adj*	*1.04 (0.31-3.55)*	*0.95*
Singletons	*>1998*	*ICSI, fresh*	*13*	*29,649*	*33.4*	*Adj*	*0.92 (0.45-1.87)*	*0.81*
Singletons	*>1998*	*ICSI, frozen*	*0*	*4,446*	*0.0*	*Adj*	*x*	*x*
Singletons	*>1998*	*ICSI, fresh, surgery*	*1*	*2,220*	*33.7*	*Adj*	*0.94 (0.12-7.09)*	*0.95*
Singletons	*>1998*	*ICSI, frozen, surgery*	*0*	*440*	*0.0*	*Adj*	*x*	*x*

Crude model denotes model adjusting for birth year, age and sex only; Adj model denotes adjusting for birth year, age and sex and additionally adjusting for paternal psychiatric history, maternal psychiatric history and paternal and maternal age; AdjC model denotes adjusting for birth year, age and sex, and additionally for calendar time; AdjI model denotes adjusting for birth year, age and sex, paternal psychiatric history, maternal psychiatric history and paternal and maternal age, and additionally for years of infertility; AdjG model denotes adjusting for birth year, age and sex and paternal psychiatric history, maternal psychiatric history and paternal and maternal age, and additionally adjusting for presence of genetic diseases.Note: x indicate cells not estimable since too few cases.

eTable 6 Mental Retardation. Comparing specific IVF procedures vs IVF without ICSI, fresh embryo

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children		IVF without ICSI, fresh	94	143,924	60.8	reference group		
All Children		IVF without ICSI, frozen	13	17,095	69.0	Crude	1.14 (0.63-2.04)	0.67
All Children						Adj	1.16 (0.64-2.07)	0.63
All Children						*AdjC*	*1.14 (0.63-2.04)*	*0.67*
All Children						*AdjI*	*1.16 (0.64-2.07)*	*0.63*
All Children						*AdjG*	*1.17 (0.65-2.09)*	*0.60*
All Children		ICSI, fresh	59	58,177	90.6	Crude	1.49 (1.05-2.11)	0.03
All Children						Adj	1.47 (1.03-2.09)	0.03
All Children						*AdjC*	*1.49 (1.05-2.11)*	*0.03*
All Children						*AdjI*	*1.47 (1.03-2.08)*	*0.03*
All Children						*AdjG*	*1.48 (1.04-2.11)*	*0.03*

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eTable 6 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children		ICSI, frozen	8	7,005	103.9	Crude	1.71 (0.82-3.58)	0.16
All Children						Adj	1.70 (0.81-3.56)	0.16
All Children						*AdjC*	*1.71 (0.82-3.58)*	*0.16*
All Children						*AdjI*	*1.70 (0.81-3.57)*	*0.16*
All Children						*AdjG*	*1.72 (0.82-3.60)*	*0.15*
All Children		ICSI, fresh, surgery	6	3,722	144.1	Crude	2.37 (1.03-5.48)	0.04
All Children						Adj	2.35 (1.01-5.45)	0.05
All Children						*AdjC*	*2.37 (1.02-5.47)*	*0.04*
All Children						*AdjI*	*2.35 (1.01-5.46)*	*0.05*
All Children						*AdjG*	*2.37 (1.02-5.51)*	*0.04*
All Children		ICSI, frozen, surgery	0	787	0.0	Crude	x	x
All Children						Adj	x	x
All Children						*AdjC*	*x*	*x*
All Children						*AdjI*	*x*	*x*
All Children						*AdjG*	*x*	*x*

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eTable 6 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*Pre-Term*	*IVF without ICSI, fresh*	*38*	*36,788*	*92.2*	*reference group*		
All Children	*Pre-Term*	*IVF without ICSI, frozen*	*5*	*3,285*	*131.0*	*Adj*	*1.44 (0.56-3.66)*	*0.45*
All Children	*Pre-Term*	*ICSI, fresh*	*19*	*11,472*	*136.1*	*Adj*	*1.46 (0.83-2.59)*	*0.19*
All Children	*Pre-Term*	*ICSI, frozen*	*4*	*1,035*	*363.0*	*Adj*	*3.47 (1.22-9.90)*	*0.02*
All Children	*Pre-Term*	*ICSI, fresh, surgery*	*4*	*768*	*413.9*	*Adj*	*4.38 (1.53-12.48)*	*<.01*
All Children	*Pre-Term*	*ICSI, frozen, surgery*	*0*	*78*	*0.0*	*Adj*	*x*	*x*
All Children	*Term*	*IVF without ICSI, fresh*	*56*	*107,136*	*49.5*	*reference group*		
All Children	*Term*	*IVF without ICSI, frozen*	*8*	*13,810*	*51.3*	*Adj*	*1.07 (0.51-2.25)*	*0.86*
All Children	*Term*	*ICSI, fresh*	*40*	*46,706*	*73.8*	*Adj*	*1.51 (0.99-2.31)*	*0.06*
All Children	*Term*	*ICSI, frozen*	*4*	*5,969*	*55.3*	*Adj*	*1.19 (0.43-3.30)*	*0.74*
All Children	*Term*	*ICSI, fresh, surgery*	*2*	*2,954*	*57.2*	*Adj*	*1.21 (0.29-4.99)*	*0.79*
All Children	*Term*	*ICSI, frozen, surgery*	*0*	*708*	*0.0*	*Adj*	*x*	*x*

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eTable 6 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*Boys*	*IVF without ICSI, fresh*	*67*	*76,251*	*89.0*	*reference group*		
All Children	*Boys*	*IVF without ICSI, frozen*	*7*	*8,785*	*75.9*	*Adj*	*0.90 (0.41-1.96)*	*0.78*
All Children	*Boys*	*ICSI, fresh*	*41*	*28,570*	*128.7*	*Adj*	*1.50 (1.00-2.26)*	*0.05*
All Children	*Boys*	*ICSI, frozen*	*5*	*3,262*	*137.3*	*Adj*	*1.61 (0.64-4.06)*	*0.31*
All Children	*Boys*	*ICSI, fresh, surgery*	*3*	*1,807*	*147.7*	*Adj*	*1.73 (0.54-5.57)*	*0.36*
All Children	*Boys*	*ICSI, frozen, surgery*	*0*	*445*	*0.0*	*Adj*	*x*	*x*
All Children	*Girls*	*IVF without ICSI, fresh*	*27*	*67,673*	*39.3*	*reference group*		
All Children	*Girls*	*IVF without ICSI, frozen*	*6*	*8,310*	*72.9*	*Adj*	*1.75 (0.72-4.25)*	*0.22*
All Children	*Girls*	*ICSI, fresh*	*18*	*29,607*	*63.0*	*Adj*	*1.41 (0.76-2.59)*	*0.28*
All Children	*Girls*	*ICSI, frozen*	*3*	*3,743*	*83.2*	*Adj*	*1.89 (0.57-6.30)*	*0.30*
All Children	*Girls*	*ICSI, fresh, surgery*	*3*	*1,915*	*163.5*	*Adj*	*3.69 (1.11-12.29)*	*0.03*
All Children	*Girls*	*ICSI, frozen, surgery*	*0*	*342*	*0.0*	*Adj*	*x*	*x*

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eTable 6 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*>1998*	*IVF without ICSI, fresh*	*36*	*52,945*	*61.2*	*reference group*		
All Children	*>1998*	*IVF without ICSI, frozen*	*4*	*8,127*	*44.7*	*Adj*	*0.74 (0.26-2.10)*	*0.57*
All Children	*>1998*	*ICSI, fresh*	*48*	*41,626*	*106.9*	*Adj*	*1.78 (1.15-2.75)*	*<.01*
All Children	*>1998*	*ICSI, frozen*	*6*	*5,629*	*98.9*	*Adj*	*1.64 (0.69-3.92)*	*0.26*
All Children	*>1998*	*ICSI, fresh, surgery*	*4*	*3,135*	*119.3*	*Adj*	*2.08 (0.74-5.89)*	*0.17*
All Children	*>1998*	*ICSI, frozen, surgery*	*0*	*639*	*0.0*	*Adj*	*x*	*x*
Singletons		IVF without ICSI, fresh	48	88,895	50.6	reference group		
Singletons		IVF without ICSI, frozen	11	12,271	83.6	Crude	1.65 (0.85-3.21)	0.14
Singletons						Adj	1.67 (0.86-3.24)	0.13
Singletons						*AdjC*	*1.66 (0.85-3.21)*	*0.14*
Singletons						*AdjI*	*1.66 (0.85-3.23)*	*0.14*
Singletons						*AdjG*	*1.70 (0.87-3.31)*	*0.12*

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eTable 6 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons		ICSI, fresh	34	39,883	80.0	Crude	1.58 (0.99-2.53)	0.06
Singletons						Adj	1.60 (1.00-2.57)	0.05
Singletons						*AdjC*	*1.58 (0.98-2.52)*	*0.06*
Singletons						*AdjI*	*1.59 (0.99-2.55)*	*0.06*
Singletons						*AdjG*	*1.63 (1.01-2.62)*	*0.04*
Singletons		ICSI, frozen	7	5,499	118.4	Crude	2.34 (1.03-5.31)	0.04
Singletons		ICSI, frozen	7	5,499	118.4	Adj	2.36 (1.04-5.36)	0.04
Singletons						*AdjC*	*2.33 (1.03-5.29)*	*0.04*
Singletons						*AdjI*	*2.37 (1.04-5.38)*	*0.04*
Singletons						*AdjG*	*2.40 (1.06-5.46)*	*0.04*
Singletons		ICSI, fresh, surgery	1	2,564	36.1	Crude	0.71 (0.10-5.22)	0.74
Singletons						Adj	0.70 (0.10-5.16)	0.73
Singletons						*AdjC*	*0.71 (0.10-5.18)*	*0.73*
Singletons						*AdjI*	*0.70 (0.10-5.14)*	*0.73*
Singletons						*AdjG*	*0.72 (0.10-5.26)*	*0.74*

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Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons		ICSI, frozen, surgery	0	566	0.0	Crude	x	x
Singletons						Adj	x	x
Singletons						*AdjC*	*x*	*x*
Singletons						*AdjI*	*x*	*x*
Singletons						*AdjG*	*x*	*x*
Singletons	*Pre-Term*	*IVF without ICSI, fresh*	*7*	*9,082*	*61.5*	*reference group*		
Singletons	*Pre-Term*	*IVF without ICSI, frozen*	*4*	*918*	*417.5*	*Adj*	*5.47 (1.58-18.96)*	*<.01*
Singletons	*Pre-Term*	*ICSI, fresh*	*3*	*3,392*	*85.3*	*Adj*	*1.18 (0.30-4.65)*	*0.81*
Singletons	*Pre-Term*	*ICSI, frozen*	*3*	*429*	*737.0*	*Adj*	*9.26 (2.35-36.56)*	*<.01*
Singletons	*Pre-Term*	*ICSI, fresh, surgery*	*0*	*215*	*0.0*	*Adj*	*x*	*x*
Singletons	*Pre-Term*	*ICSI, frozen, surgery*	*0*	*30*	*0.0*	*Adj*	*x*	*x*

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eTable 6 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons	*Term*	*IVF without ICSI, fresh*	*41*	*79,813*	*48.3*	*reference group*		
Singletons	*Term*	*IVF without ICSI, frozen*	*7*	*11,353*	*55.4*	*Adj*	*1.20 (0.53-2.70)*	*0.66*
Singletons	*Term*	*ICSI, fresh*	*31*	*36,491*	*75.7*	*Adj*	*1.66 (1.01-2.73)*	*0.05*
Singletons	*Term*	*ICSI, frozen*	*4*	*5,070*	*68.7*	*Adj*	*1.52 (0.53-4.33)*	*0.43*
Singletons	*Term*	*ICSI, fresh, surgery*	*1*	*2,348*	*37.0*	*Adj*	*0.81 (0.11-5.96)*	*0.84*
Singletons	*Term*	*ICSI, frozen, surgery*	*0*	*535*	*0.0*	*Adj*	*x*	*x*
Singletons	*Boys*	*IVF without ICSI, fresh*	*34*	*47,029*	*74.0*	*reference group*		
Singletons	*Boys*	*IVF without ICSI, frozen*	*7*	*6,352*	*106.2*	*Adj*	*1.55 (0.68-3.53)*	*0.30*
Singletons	*Boys*	*ICSI, fresh*	*22*	*19,584*	*104.8*	*Adj*	*1.54 (0.88-2.71)*	*0.13*
Singletons	*Boys*	*ICSI, frozen*	*4*	*2,571*	*136.6*	*Adj*	*2.06 (0.71-5.94)*	*0.18*
Singletons	*Boys*	*ICSI, fresh, surgery*	*1*	*1,270*	*71.6*	*Adj*	*1.02 (0.14-7.57)*	*0.98*
Singletons	*Boys*	*ICSI, frozen, surgery*	*0*	*310*	*0.0*	*Adj*	*x*	*x*

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eTable 6 (cont.)

Dataset	Sub-Group	IVF procedure	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons	*Girls*	*IVF without ICSI, fresh*	*14*	*41,865*	*31.2*	*reference group*		
Singletons	*Girls*	*IVF without ICSI, frozen*	*4*	*5,919*	*68.9*	*Adj*	*1.93 (0.63-5.90)*	*0.25*
Singletons	*Girls*	*ICSI, fresh*	*12*	*20,299*	*64.2*	*Adj*	*1.72 (0.78-3.80)*	*0.18*
Singletons	*Girls*	*ICSI, frozen*	*3*	*2,928*	*113.0*	*Adj*	*2.96 (0.84-10.46)*	*0.09*
Singletons	*Girls*	*ICSI, fresh, surgery*	*0*	*1,294*	*0.0*	*Adj*	*x*	*x*
Singletons	*Girls*	*ICSI, frozen, surgery*	*0*	*256*	*0.0*	*Adj*	*x*	*x*
Singletons	*>1998*	*IVF without ICSI, fresh*	*20*	*36,516*	*47.2*	*reference group*		
Singletons	*>1998*	*IVF without ICSI, frozen*	*4*	*6,405*	*51.9*	*Adj*	*1.07 (0.36-3.17)*	*0.90*
Singletons	*>1998*	*ICSI, fresh*	*29*	*29,601*	*87.8*	*Adj*	*1.94 (1.09-3.45)*	*0.02*
Singletons	*>1998*	*ICSI, frozen*	*6*	*4,428*	*117.4*	*Adj*	*2.58 (1.03-6.47)*	*0.04*
Singletons	*>1998*	*ICSI, fresh, surgery*	*1*	*2,214*	*41.5*	*Adj*	*0.94 (0.13-7.06)*	*0.95*
Singletons	*>1998*	*ICSI, frozen, surgery*	*0*	*440*	*0*	*Adj*	*x*	*X*

Note: x indicate cells not estimable since too few cases.

Crude model denotes model adjusting for birth year, age and sex only; Adj model denotes adjusting for birth year, age and sex and additionally adjusting for paternal psychiatric history, maternal psychiatric history and paternal and maternal age; AdjC model denotes adjusting for birth year, age and sex, and additionally for calendar time; AdjI model denotes adjusting for birth year, age and sex, paternal psychiatric history, maternal psychiatric history and paternal and maternal age, and additionally for years of infertility; AdjG model denotes adjusting for birth year, age and sex and paternal psychiatric history, maternal psychiatric history and paternal and maternal age, and additionally adjusting for presence of genetic diseases

eTable 7 Autistic disorder. Comparing IVF techniques.

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*Blastocyst*		*<5 days culture*	*101*	*227,184*	*32.3*		*reference group*	
All Children	*Blastocyst*		*Blastocyst*	*2*	*3,934*	*31.0*	*Crude*	*1.24 (0.29-5.31)*	*0.77*
All Children	*Blastocyst*						*Adj*	*1.40 (0.32-6.13)*	*0.66*
All Children	*Blastocyst*						*AdjI*	*1.39 (0.32-6.08)*	*0.67*
All Children	*Blastocyst*						*AdjG*	*1.40 (0.32-6.13)*	*0.66*
All Children	*Blastocyst*	*Boys*	*<5 days culture*	*75*	*117,333*	*50.2*		*reference group*	
All Children	*Blastocyst*	*Boys*	*Blastocyst*	*1*	*2,031*	*31.5*	*Adj*	*0.92 (0.12-7.08)*	*0.94*
All Children	*Blastocyst*	*Girls*	*<5 days culture*	*26*	*109,851*	*21.4*		*reference group*	
All Children	*Blastocyst*	*Girls*	*Blastocyst*	*1*	*1,902*	*46.3*	*Adj*	*2.90 (0.33-25.23)*	*0.34*
All Children	*Blastocyst*	*PreTerm*	*<5 days culture*	*35*	*52,893*	*47.1*		*reference group*	
All Children	*Blastocyst*	*PreTerm*	*Blastocyst*	*0*	*736*	*0.0*	*Adj*	*x*	*x*
All Children	*Blastocyst*	*Term*	*<5 days culture*	*66*	*174,291*	*27.8*		*reference group*	
All Children	*Blastocyst*	*Term*	*Blastocyst*	*2*	*3,198*	*37.7*	*Adj*	*2.06 (0.46-9.24)*	*0.35*

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eTable 7 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*Frozen embryo*		*Fresh*	*92*	*206,189*	*32.5*		*reference group*	
All Children	*Frozen embryo*		*Frozen*	*11*	*24,929*	*30.1*	*Crude*	*0.93 (0.49-1.74)*	*0.81*
All Children	*Frozen embryo*					*30.1*	*Adj*	*0.93 (0.50-1.75)*	*0.82*
All Children	*Frozen embryo*					*30.1*	*AdjI*	*0.93 (0.50-1.75)*	*0.83*
All Children	*Frozen embryo*					*30.1*	*AdjG*	*0.93 (0.50-1.75)*	*0.82*
All Children	*Frozen embryo*	*Boys*	*Fresh*	*69*	*106,861*	*51.0*		*reference group*	
All Children	*Frozen embryo*	*Boys*	*Frozen*	*7*	*12,504*	*40.3*	*Adj*	*0.80 (0.37-1.76)*	*0.59*
All Children	*Frozen embryo*	*Girls*	*Fresh*	*23*	*99,328*	*21.0*		*reference group*	
All Children	*Frozen embryo*	*Girls*	*Frozen*	*4*	*12,426*	*28.1*	*Adj*	*1.28 (0.44-3.72)*	*0.64*
All Children	*Frozen embryo*	*PreTerm*	*Fresh*	*32*	*49,199*	*46.2*		*reference group*	
All Children	*Frozen embryo*	*PreTerm*	*Frozen*	*3*	*4,430*	*44.7*	*Adj*	*0.91 (0.28-2.97)*	*0.87*
All Children	*Frozen embryo*	*Term*	*Fresh*	*60*	*156,990*	*28.2*		*reference group*	
All Children	*Frozen embryo*	*Term*	*Frozen*	*8*	*20,499*	*26.9*	*Adj*	*0.97 (0.46-2.03)*	*0.93*

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eTable 7 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*ICSI*		*IVF*	*63*	*161,328*	*30.7*		*reference group*	
All Children	*ICSI*		*ICSI*	*40*	*69,790*	*36.4*	*Crude*	*1.19 (0.78-1.80)*	*0.42*
All Children	*ICSI*						*Adj*	*1.21 (0.80-1.85)*	*0.37*
All Children	*ICSI*						*AdjI*	*1.21 (0.80-1.85)*	*0.37*
All Children	*ICSI*						*AdjG*	*1.21 (0.80-1.85)*	*0.37*
All Children	*ICSI*	*Boys*	*IVF*	*48*	*85,208*	*48.8*		*reference group*	
All Children	*ICSI*	*Boys*	*ICSI*	*28*	*34,156*	*52.6*	*Adj*	*1.16 (0.71-1.88)*	*0.55*
All Children	*ICSI*	*Girls*	*IVF*	*15*	*76,120*	*18.9*		*reference group*	
All Children	*ICSI*	*Girls*	*ICSI*	*12*	*35,634*	*28.8*	*Adj*	*1.37 (0.64-2.97)*	*0.42*
All Children	*ICSI*	*PreTerm*	*IVF*	*20*	*40,224*	*40.5*		*reference group*	
All Children	*ICSI*	*PreTerm*	*ICSI*	*15*	*13,405*	*64.6*	*Adj*	*1.73 (0.87-3.45)*	*0.12*
All Children	*ICSI*	*Term*	*IVF*	*43*	*121,104*	*27.8*		*reference group*	
All Children	*ICSI*	*Term*	*ICSI*	*25*	*56,385*	*28.5*	*Adj*	*1.04 (0.62-1.73)*	*0.89*

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eTable 7 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*Surgically extracted*		*Ejaculated*	*95*	*226,611*	*30.9*		*reference group*	
All Children	*Surgically extracted*		*Surgical*	*8*	*4,507*	*110.1*	*Crude*	*3.29 (1.59-6.84)*	*<.01*
All Children	*Surgically extracted*						*Adj*	*3.29 (1.58-6.87)*	*<.01*
All Children	*Surgically extracted*						*AdjI*	*3.30 (1.58-6.88)*	*<.01*
All Children	*Surgically extracted*						*AdjG*	*3.29 (1.58-6.87)*	*<.01*
All Children	*Surgically extracted*	*Boys*	*Ejaculated*	*71*	*117,114*	*48.3*		*reference group*	
All Children	*Surgically extracted*	*Boys*	*Surgical*	*5*	*2,251*	*141.1*	*Adj*	*2.78 (1.11-6.99)*	*0.03*
All Children	*Surgically extracted*	*Girls*	*Ejaculated*	*24*	*109,497*	*20.1*		*reference group*	
All Children	*Surgically extracted*	*Girls*	*Surgical*	*3*	*2,256*	*111.4*	*Adj*	*4.72 (1.39-16.04)*	*0.01*
All Children	*Surgically extracted*	*PreTerm*	*Ejaculated*	*30*	*52,787*	*42.3*		*reference group*	
All Children	*Surgically extracted*	*PreTerm*	*Surgical*	*5*	*842*	*319.8*	*Adj*	*8.06 (2.97-21.85)*	*<.01*
All Children	*Surgically extracted*	*Term*	*Ejaculated*	*65*	*173,824*	*27.6*		*reference group*	
All Children	*Surgically extracted*	*Term*	*Surgical*	*3*	*3,665*	*51.6*	*Adj*	*1.65 (0.52-5.31)*	*0.40*

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eTable 7 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons	*Blastocyst*		*<5 days culture*	*52*	*146,799*	*23.8*		*reference group*	
Singletons	*Blastocyst*		*Blastocyst*	*2*	*3,133*	*38.6*	*Crude*	*1.79 (0.41-7.92)*	*0.44*
Singletons	*Blastocyst*						*Adj*	*1.82 (0.40-8.22)*	*0.44*
Singletons	*Blastocyst*						*AdjI*	*1.84 (0.40-8.32)*	*0.43*
Singletons	*Blastocyst*						*AdjG*	*1.82 (0.40-8.22)*	*0.44*
Singletons	*Blastocyst*	*Boys*	*<5 days culture*	*41*	*75,629*	*46.0*		*reference group*	
Singletons	*Blastocyst*	*Boys*	*Blastocyst*	*1*	*1,632*	*42.0*	*Adj*	*1.04 (0.13-8.17)*	*0.97*
Singletons	*Blastocyst*	*Girls*	*<5 days culture*	*11*	*71,169*	*6.6*		*reference group*	
Singletons	*Blastocyst*	*Girls*	*Blastocyst*	*1*	*1,501*	*51.1*	*Adj*	*6.89 (0.61-77.37)*	*0.12*
Singletons	*Blastocyst*	*PreTerm*	*<5 days culture*	*7*	*13,835*	*26.9*		*reference group*	
Singletons	*Blastocyst*	*PreTerm*	*Blastocyst*	*0*	*304*	*0.0*	*Adj*	*x*	*x*
Singletons	*Blastocyst*	*Term*	*<5 days culture*	*45*	*132,964*	*20.9*		*reference group*	
Singletons	*Blastocyst*	*Term*	*Blastocyst*	*2*	*2,829*	*41.2*	*Adj*	*2.09 (0.46-9.52)*	*0.34*

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eTable 7 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons	*Frozen embryo*		*Fresh*	*49*	*131,538*	*25.1*		*reference group*	
Singletons	*Frozen embryo*		*Frozen*	*5*	*18,394*	*17.3*	*Crude*	*0.69 (0.27-1.73)*	*0.43*
Singletons	*Frozen embryo*						*Adj*	*0.71 (0.28-1.78)*	*0.46*
Singletons	*Frozen embryo*						*AdjI*	*0.71 (0.28-1.79)*	*0.47*
Singletons	*Frozen embryo*						*AdjG*	*0.71 (0.28-1.78)*	*0.46*
Singletons	*Frozen embryo*	*Boys*	*Fresh*	*39*	*68,002*	*48.8*		*reference group*	
Singletons	*Frozen embryo*	*Boys*	*Frozen*	*3*	*9,259*	*25.1*	*Adj*	*0.54 (0.17-1.76)*	*0.31*
Singletons	*Frozen embryo*	*Girls*	*Fresh*	*10*	*63,535*	*7.4*		*reference group*	
Singletons	*Frozen embryo*	*Girls*	*Frozen*	*2*	*9,135*	*10.3*	*Adj*	*1.30 (0.28-5.93)*	*0.74*
Singletons	*Frozen embryo*	*PreTerm*	*Fresh*	*7*	*12,721*	*29.3*		*reference group*	
Singletons	*Frozen embryo*	*PreTerm*	*Frozen*	*0*	*1,418*	*0.0*	*Adj*	*x*	*x*
Singletons	*Frozen embryo*	*Term*	*Fresh*	*42*	*118,817*	*22.0*		*reference group*	
Singletons	*Frozen embryo*	*Term*	*Frozen*	*5*	*16,977*	*17.4*	*Adj*	*0.82 (0.32-2.08)*	*0.68*

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eTable 7 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons	*ICSI*		*IVF*	*35*	*101,347*	*24.6*		*reference group*	
Singletons	*ICSI*		*ICSI*	*19*	*48,585*	*23.1*	*Crude*	*0.94 (0.53-1.67)*	*0.83*
Singletons	*ICSI*						*Adj*	*1.01 (0.56-1.81)*	*0.98*
Singletons	*ICSI*						*AdjI*	*1.00 (0.56-1.79)*	*1.00*
Singletons	*ICSI*						*AdjG*	*1.01 (0.56-1.81)*	*0.98*
Singletons	*ICSI*	*Boys*	*IVF*	*28*	*53,475*	*47.3*		*reference group*	
Singletons	*ICSI*	*Boys*	*ICSI*	*14*	*23,786*	*42.3*	*Adj*	*0.97 (0.50-1.88)*	*0.92*
Singletons	*ICSI*	*Girls*	*IVF*	*7*	*47,872*	*7.7*		*reference group*	
Singletons	*ICSI*	*Girls*	*ICSI*	*5*	*24,798*	*8.0*	*Adj*	*1.15 (0.36-3.68)*	*0.81*
Singletons	*ICSI*	*PreTerm*	*IVF*	*6*	*10,050*	*32.5*		*reference group*	
Singletons	*ICSI*	*PreTerm*	*ICSI*	*1*	*4,089*	*12.0*	*Adj*	*0.35 (0.04-2.91)*	*0.33*
Singletons	*ICSI*	*Term*	*IVF*	*29*	*91,297*	*21.3*		*reference group*	
Singletons	*ICSI*	*Term*	*ICSI*	*18*	*44,496*	*21.7*	*Adj*	*1.13 (0.62-2.09)*	*0.69*

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eTable 7 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons	*Surgically extracted*		*Ejaculated*	*53*	*146,797*	*24.3*		*reference group*	
Singletons	*Surgically extracted*		*Surgical*	*1*	*3,135*	*18.3*	*Crude*	*0.68 (0.09-4.94)*	*0.70*
Singletons	*Surgically extracted*		*Surgical*	*1*	*3,135*	*18.3*	*Adj*	*0.73 (0.10-5.30)*	*0.75*
Singletons	*Surgically extracted*						*AdjI*	*0.72 (0.10-5.25)*	*0.75*
Singletons	*Surgically extracted*						*AdjG*	*0.73 (0.10-5.30)*	*0.75*
Singletons	*Surgically extracted*	*Boys*	*Ejaculated*	*41*	*75,676*	*45.9*		*reference group*	
Singletons	*Surgically extracted*	*Boys*	*Surgical*	*1*	*1,585*	*45.0*	*Adj*	*0.96 (0.13-7.02)*	*0.97*
Singletons	*Surgically extracted*	*Girls*	*Ejaculated*	*12*	*71,121*	*7.9*		*reference group*	
Singletons	*Surgically extracted*	*Girls*	*Surgical*	*0*	*1,550*	*0.0*	*Adj*	*x*	*x*
Singletons	*Surgically extracted*	*PreTerm*	*Ejaculated*	*7*	*13,893*	*26.8*		*reference group*	
Singletons	*Surgically extracted*	*PreTerm*	*Surgical*	*0*	*246*	*0.0*	*Adj*	*x*	*x*
Singletons	*Surgically extracted*	*Term*	*Ejaculated*	*46*	*132,904*	*21.5*		*reference group*	
Singletons	*Surgically extracted*	*Term*	*Surgical*	*1*	*2,889*	*17.8*	*Adj*	*0.83 (0.11-6.03)*	*0.85*

Note: x indicate cells not estimable since too few cases. Crude model denotes model adjusting for birth year, age and sex only; Adj model denotes adjusting for birth year, age and sex and additionally adjusting for paternal psychiatric history, maternal psychiatric history and paternal and maternal age; AdjC model denotes adjusting for birth year, age and sex, and additionally for calendar time; AdjI model denotes adjusting for birth year, age and sex, paternal psychiatric history, maternal psychiatric history and paternal and maternal age, and additionally for years of infertility; AdjG model denotes adjusting for birth year, age and sex and paternal psychiatric history, maternal psychiatric history and paternal and maternal age, and additionally adjusting for presence of genetic diseases

eTable 8 Mental Retardation. Comparing IVF techniques.

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*Blastocyst*		*<5 days culture*	*179*	*226,774*	*71.9*		*reference group*	
All Children	*Blastocyst*		*Blastocyst*	*1*	*3,936*	*19.5*	*Crude*	*0.27 (0.04-1.94)*	*0.19*
All Children	*Blastocyst*						*Adj*	*0.28 (0.04-2.01)*	*0.20*
All Children	*Blastocyst*						*AdjI*	*0.27 (0.04-2.00)*	*0.20*
All Children	*Blastocyst*						*AdjG*	*0.28 (0.04-2.01)*	*0.20*
All Children	*Blastocyst*	*Boys*	*<5 days culture*	*122*	*117,089*	*100.0*		*reference group*	
All Children	*Blastocyst*	*Boys*	*Blastocyst*	*1*	*2,031*	*39.6*	*Adj*	*0.42 (0.06-3.11)*	*0.40*
All Children	*Blastocyst*	*Girls*	*<5 days culture*	*57*	*109,685*	*52.0*		*reference group*	
All Children	*Blastocyst*	*Girls*	*Blastocyst*	*0*	*1,904*	*0.0*	*Adj*	*x*	*x*
All Children	*Blastocyst*	*PreTerm*	*<5 days culture*	*70*	*52,691*	*115.4*		*reference group*	
All Children	*Blastocyst*	*PreTerm*	*Blastocyst*	*0*	*736*	*0.0*	*Adj*	*x*	*x*
All Children	*Blastocyst*	*Term*	*<5 days culture*	*109*	*174,083*	*56.7*		*reference group*	
All Children	*Blastocyst*	*Term*	*Blastocyst*	*1*	*3,200*	*19.7*	*Adj*	*0.36 (0.05-2.67)*	*0.32*

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*Frozen embryo*		*Fresh*	*159*	*205,824*	*70.5*		*reference group*	
All Children	*Frozen embryo*		*Frozen*	*21*	*24,886*	*74.2*	*Crude*	*1.05 (0.67-1.66)*	*0.83*
All Children	*Frozen embryo*						*Adj*	*1.07 (0.68-1.69)*	*0.77*
All Children	*Frozen embryo*						*AdjI*	*1.07 (0.68-1.70)*	*0.77*
All Children	*Frozen embryo*						*AdjG*	*1.08 (0.68-1.70)*	*0.75*
All Children	*Frozen embryo*	*Boys*	*Fresh*	*111*	*106,629*	*100.3*		*reference group*	
All Children	*Frozen embryo*	*Boys*	*Frozen*	*12*	*12,492*	*86.9*	*Adj*	*0.90 (0.49-1.63)*	*0.72*
All Children	*Frozen embryo*	*Girls*	*Fresh*	*48*	*99,195*	*48.5*		*reference group*	
All Children	*Frozen embryo*	*Girls*	*Frozen*	*9*	*12,394*	*71.7*	*Adj*	*1.45 (0.71-2.97)*	*0.30*
All Children	*Frozen embryo*	*PreTerm*	*Fresh*	*61*	*49,028*	*107.6*		*reference group*	
All Children	*Frozen embryo*	*PreTerm*	*Frozen*	*9*	*4,399*	*174.7*	*Adj*	*1.56 (0.77-3.15)*	*0.22*
All Children	*Frozen embryo*	*Term*	*Fresh*	*98*	*156,795*	*56.9*		*reference group*	
All Children	*Frozen embryo*	*Term*	*Frozen*	*12*	*20,488*	*49.2*	*Adj*	*0.90 (0.49-1.64)*	*0.73*

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Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*ICSI*		*IVF*	*107*	*161,019*	*61.8*		*reference group*	
All Children	*ICSI*		*ICSI*	*73*	*69,691*	*93.5*	*Crude*	*1.51 (1.10-2.09)*	*0.01*
All Children	*ICSI*						*Adj*	*1.51 (1.09-2.09)*	*0.01*
All Children	*ICSI*						*AdjI*	*1.51 (1.09-2.08)*	*0.01*
All Children	*ICSI*						*AdjG*	*1.52 (1.10-2.11)*	*0.01*
All Children	*ICSI*	*Boys*	*IVF*	*74*	*85,036*	*87.6*		*reference group*	
All Children	*ICSI*	*Boys*	*ICSI*	*49*	*34,084*	*129.4*	*Adj*	*1.54 (1.05-2.25)*	*0.03*
All Children	*ICSI*	*Girls*	*IVF*	*33*	*75,982*	*43.2*		*reference group*	
All Children	*ICSI*	*Girls*	*ICSI*	*24*	*35,607*	*68.8*	*Adj*	*1.45 (0.85-2.49)*	*0.18*
All Children	*ICSI*	*PreTerm*	*IVF*	*43*	*40,073*	*96.0*		*reference group*	
All Children	*ICSI*	*PreTerm*	*ICSI*	*27*	*13,354*	*166.7*	*Adj*	*1.73 (1.05-2.86)*	*0.03*
All Children	*ICSI*	*Term*	*IVF*	*64*	*120,946*	*49.8*		*reference group*	
All Children	*ICSI*	*Term*	*ICSI*	*46*	*56,337*	*70.1*	*Adj*	*1.44 (0.97-2.15)*	*0.07*

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eTable 8 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
All Children	*Surgically extracted*		*Ejaculated*	*174*	*226,201*	*70.1*		*reference group*	
All Children	*Surgically extracted*		*Surgical*	*6*	*4,508*	*112.1*	*Crude*	*1.56 (0.69-3.54)*	*0.29*
All Children	*Surgically extracted*						*Adj*	*1.67 (0.73-3.79)*	*0.22*
All Children	*Surgically extracted*						*AdjI*	*1.67 (0.73-3.79)*	*0.22*
All Children	*Surgically extracted*						*AdjG*	*1.68 (0.74-3.83)*	*0.22*
All Children	*Surgically extracted*	*Boys*	*Ejaculated*	*120*	*116,869*	*98.6*		*reference group*	
All Children	*Surgically extracted*	*Boys*	*Surgical*	*3*	*2,252*	*112.9*	*Adj*	*1.19 (0.38-3.78)*	*0.76*
All Children	*Surgically extracted*	*Girls*	*Ejaculated*	*54*	*109,333*	*49.4*		*reference group*	
All Children	*Surgically extracted*	*Girls*	*Surgical*	*3*	*2,256*	*130.2*	*Adj*	*2.77 (0.85-9.02)*	*0.09*
All Children	*Surgically extracted*	*PreTerm*	*Ejaculated*	*66*	*52,580*	*109.4*		*reference group*	
All Children	*Surgically extracted*	*PreTerm*	*Surgical*	*4*	*846*	*356.7*	*Adj*	*3.31 (1.18-9.31)*	*0.02*
All Children	*Surgically extracted*	*Term*	*Ejaculated*	*108*	*173,621*	*56.2*		*reference group*	
All Children	*Surgically extracted*	*Term*	*Surgical*	*2*	*3,662*	*43.5*	*Adj*	*0.83 (0.20-3.40)*	*0.80*

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eTable 8 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons	*Blastocyst*		*<5 days culture*	*100*	*146,542*	*63.7*		*reference group*	
Singletons	*Blastocyst*		*Blastocyst*	*1*	*3,135*	*23.7*	*Crude*	*0.39 (0.05-2.85)*	*0.35*
Singletons	*Blastocyst*						*Adj*	*0.35 (0.05-2.61)*	*0.31*
Singletons	*Blastocyst*						*AdjI*	*0.36 (0.05-2.65)*	*0.31*
Singletons	*Blastocyst*						*AdjG*	*0.35 (0.05-2.61)*	*0.31*
Singletons	*Blastocyst*	*Boys*	*<5 days culture*	*67*	*75,485*	*86.9*		*reference group*	
Singletons	*Blastocyst*	*Boys*	*Blastocyst*	*1*	*1,632*	*43.2*	*Adj*	*0.46 (0.06-3.42)*	*0.45*
Singletons	*Blastocyst*	*Girls*	*<5 days culture*	*33*	*71,057*	*46.4*		*reference group*	
Singletons	*Blastocyst*	*Girls*	*Blastocyst*	*0*	*1,503*	*0.0*	*Adj*	*x*	*x*
Singletons	*Blastocyst*	*PreTerm*	*<5 days culture*	*17*	*13,764*	*111.3*		*reference group*	
Singletons	*Blastocyst*	*PreTerm*	*Blastocyst*	*0*	*304*	*0.0*	*Adj*	*x*	*x*
Singletons	*Blastocyst*	*Term*	*<5 days culture*	*83*	*132,779*	*57.1*		*reference group*	
Singletons	*Blastocyst*	*Term*	*Blastocyst*	*1*	*2,831*	*24.4*	*Adj*	*0.39 (0.05-2.86)*	*0.35*

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eTable 8 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons	*Frozen embryo*		*Fresh*	*83*	*131,341*	*59.1*		*reference group*	
Singletons	*Frozen embryo*		*Frozen*	*18*	*18,336*	*89.1*	*Crude*	*1.51 (0.90-2.52)*	*0.12*
Singletons	*Frozen embryo*						*Adj*	*1.52 (0.91-2.55)*	*0.11*
Singletons	*Frozen embryo*						*AdjI*	*1.52 (0.91-2.55)*	*0.11*
Singletons	*Frozen embryo*						*AdjG*	*1.53 (0.92-2.57)*	*0.10*
Singletons	*Frozen embryo*	*Boys*	*Fresh*	*57*	*67,883*	*82.8*		*reference group*	
Singletons	*Frozen embryo*	*Boys*	*Frozen*	*11*	*9,233*	*108.8*	*Adj*	*1.38 (0.72-2.66)*	*0.33*
Singletons	*Frozen embryo*	*Girls*	*Fresh*	*26*	*63,458*	*40.7*		*reference group*	
Singletons	*Frozen embryo*	*Girls*	*Frozen*	*7*	*9,102*	*78.5*	*Adj*	*1.80 (0.78-4.16)*	*0.17*
Singletons	*Frozen embryo*	*PreTerm*	*Fresh*	*10*	*12,690*	*66.9*		*reference group*	
Singletons	*Frozen embryo*	*PreTerm*	*Frozen*	*7*	*1,378*	*498.0*	*Adj*	*6.02 (2.27-15.96)*	*<.01*
Singletons	*Frozen embryo*	*Term*	*Fresh*	*73*	*118,652*	*56.4*		*reference group*	
Singletons	*Frozen embryo*	*Term*	*Frozen*	*11*	*16,958*	*56.1*	*Adj*	*1.03 (0.54-1.95)*	*0.92*

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eTable 8 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons	*ICSI*		*IVF*	*59*	*101,166*	*54.8*		*reference group*	
Singletons	*ICSI*		*ICSI*	*42*	*48,511*	*80.2*	*Crude*	*1.46 (0.96-2.23)*	*0.08*
Singletons	*ICSI*						*Adj*	*1.50 (0.98-2.29)*	*0.06*
Singletons	*ICSI*						*AdjI*	*1.49 (0.97-2.28)*	*0.07*
Singletons	*ICSI*						*AdjG*	*1.52 (0.99-2.33)*	*0.06*
Singletons	*ICSI*	*Boys*	*IVF*	*41*	*53,381*	*78.2*		*reference group*	
Singletons	*ICSI*	*Boys*	*ICSI*	*27*	*23,735*	*104.3*	*Adj*	*1.46 (0.88-2.44)*	*0.15*
Singletons	*ICSI*	*Girls*	*IVF*	*18*	*47,784*	*35.9*		*reference group*	
Singletons	*ICSI*	*Girls*	*ICSI*	*15*	*24,776*	*64.9*	*Adj*	*1.57 (0.78-3.17)*	*0.21*
Singletons	*ICSI*	*PreTerm*	*IVF*	*11*	*10,000*	*97.4*		*reference group*	
Singletons	*ICSI*	*PreTerm*	*ICSI*	*6*	*4,067*	*136.2*	*Adj*	*1.36 (0.49-3.75)*	*0.55*
Singletons	*ICSI*	*Term*	*IVF*	*48*	*91,165*	*49.3*		*reference group*	
Singletons	*ICSI*	*Term*	*ICSI*	*36*	*44,444*	*71.8*	*Adj*	*1.53 (0.97-2.42)*	*0.07*

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eTable 8 (cont.)

Dataset	Technique	Sub-Group	Group of children	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Singletons	*Surgically extracted*		*Ejaculated*	*100*	*146,548*	*63.5*		*reference group*	
Singletons	*Surgically extracted*		*Surgical*	*1*	*3,129*	*27.6*	*Crude*	*0.42 (0.06-3.06)*	*0.40*
Singletons	*Surgically extracted*						*Adj*	*0.45 (0.06-3.22)*	*0.42*
Singletons	*Surgically extracted*						*AdjI*	*0.45 (0.06-3.22)*	*0.42*
Singletons	*Surgically extracted*						*AdjG*	*0.45 (0.06-3.27)*	*0.43*
Singletons	*Surgically extracted*	*Boys*	*Ejaculated*	*67*	*75,537*	*86.5*		*reference group*	
Singletons	*Surgically extracted*	*Boys*	*Surgical*	*1*	*1,580*	*54.5*	*Adj*	*0.64 (0.09-4.65)*	*0.66*
Singletons	*Surgically extracted*	*Girls*	*Ejaculated*	*33*	*71,011*	*46.4*		*reference group*	
Singletons	*Surgically extracted*	*Girls*	*Surgical*	*0*	*1,550*	*0.0*	*Adj*	*x*	*x*
Singletons	*Surgically extracted*	*PreTerm*	*Ejaculated*	*17*	*13,822*	*110.5*		*reference group*	
Singletons	*Surgically extracted*	*PreTerm*	*Surgical*	*0*	*246*	*0.0*	*Adj*	*x*	*x*
Singletons	*Surgically extracted*	*Term*	*Ejaculated*	*83*	*132,726*	*57.0*		*reference group*	
Singletons	*Surgically extracted*	*Term*	*Surgical*	*1*	*2,884*	*28.4*	*Adj*	*0.53 (0.07-3.85)*	*0.53*

Crude model denotes model adjusting for birth year, age and sex only; Adj model denotes adjusting for birth year, age and sex and additionally adjusting for paternal psychiatric history, maternal psychiatric history and paternal and maternal age; AdjC model denotes adjusting for birth year, age and sex, and additionally for calendar time; AdjI model denotes adjusting for birth year, age and sex, paternal psychiatric history, maternal psychiatric history and paternal and maternal age, and additionally for years of infertility; AdjG model denotes adjusting for birth year, age and sex and paternal psychiatric history, maternal psychiatric history and paternal and maternal age, and additionally adjusting for presence of genetic diseases. Note: x indicate cells not estimable since too few cases.

eTable 9 Hormones. Comparing children born following hormone treatment as only fertility treatment vs children spontaneous conceived without use of hormones.

Dataset	Hormones or No Hormones	Number of Cases	Person Years	Rate per 100,000	Model	RR (95% CI)	p-value
Autistic Disorder							
All Children	*No Hormones*	*6,933*	*34,155,462*	*15.6*	*reference group*		
All Children	*Hormone treated*	*26*	*70,333*	*14.1*	*Crude*	*0.90 (0.61-1.33)*	*0.61*
All Children					*Adj*	*0.91 (0.62-1.34)*	*0.64*
Singletons	*No Hormones*	*6,713*	*33,372,926*	*15.0*	*reference group*		
Singletons	*Hormone treated*	*24*	*62,389*	*14.2*	*Crude*	*0.95 (0.63-1.42)*	*0.79*
Singletons					*Adj*	*0.96 (0.64-1.43)*	*0.84*
Mental Retardation							
All Children	*No Hormones*	*15,784*	*34,108,394*	*39.8*	*reference group*		
All Children	*Hormone treated*	*43*	*70,277*	*34.4*	*Crude*	*0.86 (0.64-1.16)*	*0.34*
All Children					*Adj*	*0.89 (0.66-1.21)*	*0.46*
Singletons	*No Hormones*	*15,241*	*33,327,569*	*38.5*	*reference group*		
Singletons	*Hormone treated*	*38*	*62,342*	*33.4*	*Crude*	*0.87 (0.63-1.19)*	*0.39*
Singletons					*Adj*	*0.90 (0.65-1.24)*	*0.52*

Crude model denotes model adjusting for birth year, age and sex only; Adj model denotes adjusting for birth year, age and sex and additionally adjusting for paternal psychiatric history, maternal psychiatric history and paternal and maternal age. Note: x indicate cells not estimable since too few cases.

eTable 10 Comparisons of children born following specific IVF procedures vs children born after spontaneous conception.

Dataset	IVF Procedure	Sub-Group	Autistic Disorder RR (95% CI)#	Autistic Disorder p-value	Mental Retardation RR (95% CI)#	Mental Retardation p-value
All Children	IVF without ICSI, fresh		1.01 (0.77 - 1.32)	0.96	1.01 (0.83 - 1.24)	0.91
All Children	*IVF without ICSI, fresh*	*>1998##*	*1.14 (0.79 - 1.63)*	*0.49*	*0.92 (0.66 - 1.28)*	*0.64*
All Children	*IVF without ICSI, fresh*	*Boys*	*1.07 (0.79 - 1.45)*	*0.66*	*1.14 (0.90 - 1.45)*	*0.28*
All Children	*IVF without ICSI, fresh*	*Girls*	*0.85 (0.47 - 1.54)*	*0.60*	*0.75 (0.51 - 1.09)*	*0.13*
All Children	*IVF without ICSI, fresh*	*Pre-Term*	*0.82 (0.51 - 1.33)*	*0.42*	*0.69 (0.50 - 0.95)*	*0.02*
All Children	*IVF without ICSI, fresh*	*Term*	*0.95 (0.68 - 1.32)*	*0.75*	*0.86 (0.66 - 1.12)*	*0.25*
All Children	IVF without ICSI, frozen		1.43 (0.77- 2.66)	0.26	1.12 (0.65- 1.93)	0.68
All Children	*IVF without ICSI, frozen*	*>1998##*	*1.25 (0.52- 3.00)*	*0.62*	*0.67 (0.25- 1.79)*	*0.42*
All Children	*IVF without ICSI, frozen*	*Boys*	*1.19 (0.54- 2.66)*	*0.67*	*1.00 (0.48- 2.10)*	*1.00*
All Children	*IVF without ICSI, frozen*	*Girls*	*2.17 (0.81- 5.80)*	*0.12*	*1.26 (0.56- 2.80)*	*0.58*
All Children	*IVF without ICSI, frozen*	*Pre-Term*	*1.45 (0.47- 4.51)*	*0.52*	*0.96 (0.40- 2.30)*	*0.92*
All Children	*IVF without ICSI, frozen*	*Term*	*1.30 (0.62- 2.73)*	*0.49*	*0.91 (0.46- 1.83)*	*0.80*

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eTable 10 (cont.)

Dataset	IVF Procedure	Sub-Group	Autistic Disorder RR (95% CI)#	Autistic Disorder p-value	Intellectual Disability RR (95% CI)#	Intellectual Disability p-value
All Children	ICSI, fresh		1.23 (0.86- 1.75)	0.25	1.45 (1.12- 1.87)	<.01
All Children	*ICSI, fresh*	*>1998##*	*1.27 (0.85- 1.88)*	*0.24*	*1.57 (1.18- 2.09)*	*<.01*
All Children	*ICSI, fresh*	*Boys*	*1.21 (0.79- 1.84)*	*0.38*	*1.70 (1.25- 2.31)*	*<.01*
All Children	*ICSI, fresh*	*Girls*	*1.30 (0.67- 2.50)*	*0.44*	*1.04 (0.66- 1.66)*	*0.86*
All Children	*ICSI, fresh*	*Pre-Term*	*1.28 (0.68- 2.38)*	*0.45*	*1.01 (0.64- 1.59)*	*0.96*
All Children	*ICSI, fresh*	*Term*	*1.07 (0.70- 1.65)*	*0.75*	*1.31 (0.96- 1.78)*	*0.09*
All Children	ICSI, frozen		0.32 (0.04- 2.24)	0.25	1.65 (0.82- 3.30)	0.16
All Children	*ICSI, frozen*	*>1998##*	*0.37 (0.05- 2.65)*	*0.32*	*1.48 (0.66- 3.29)*	*0.34*
All Children	*ICSI, frozen*	*Boys*	*0.45 (0.06- 3.17)*	*0.42*	*1.81 (0.75- 4.36)*	*0.18*
All Children	*ICSI, frozen*	*Girls*	*x*	*x*	*1.39 (0.45- 4.30)*	*0.57*
All Children	*ICSI, frozen*	*Pre-Term*	*x*	*x*	*2.39 (0.89- 6.37)*	*0.08*
All Children	*ICSI, frozen*	*Term*	*0.39 (0.05- 2.75)*	*0.34*	*1.03 (0.39- 2.76)*	*0.95*

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eTable 10 (cont.)

Dataset	IVF Procedure	Sub-Group	Autistic Disorder RR (95% CI)#	Autistic Disorder p-value	Intellectual Disability RR (95% CI)#	Intellectual Disability p-value
All Children	ICSI, fresh, surgery		4.56 (2.28- 9.13)	<.01	2.18 (0.98- 4.86)	0.06
All Children	*ICSI, fresh, surgery*	*>1998##*	*4.47 (2.13- 9.40)*	*<.01*	*1.67 (0.63- 4.45)*	*0.31*
All Children	*ICSI, fresh, surgery*	*Boys*	*3.92 (1.63- 9.44)*	*<.01*	*1.85 (0.59- 5.73)*	*0.29*
All Children	*ICSI, fresh, surgery*	*Girls*	*6.18 (1.99-19.18)*	*<.01*	*2.59 (0.83- 8.02)*	*0.10*
All Children	*ICSI, fresh, surgery*	*Pre-Term*	*8.08 (3.35-19.49)*	*<.01*	*2.80 (1.05- 7.48)*	*0.04*
All Children	*ICSI, fresh, surgery*	*Term*	*2.26 (0.73- 7.01)*	*0.16*	*1.00 (0.25- 4.01)*	*1.00*
All Children	ICSI, frozen, surgery		*Not estimable - Too few cases*			

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eTable 10 (cont.)

Dataset	IVF Procedure	Sub-Group	Autistic Disorder RR (95% CI)#	Autistic Disorder p-value	Intellectual Disability RR (95% CI)#	Intellectual Disability p-value
Singletons	*IVF without ICSI, fresh*		*0.89 (0.62 - 1.27)*	*0.52*	*0.83 (0.63 - 1.11)*	*0.21*
Singletons	*IVF without ICSI, fresh*	*>1998##*	*1.02 (0.65 - 1.61)*	*0.92*	*0.74 (0.48 - 1.15)*	*0.18*
Singletons	*IVF without ICSI, fresh*	*Boys*	*1.00 (0.67 - 1.48)*	*0.98*	*0.94 (0.67 - 1.31)*	*0.70*
Singletons	*IVF without ICSI, fresh*	*Girls*	*0.61 (0.25 - 1.46)*	*0.27*	*0.62 (0.37 - 1.05)*	*0.08*
Singletons	*IVF without ICSI, fresh*	*Pre-Term*	*1.01 (0.45 - 2.26)*	*0.98*	*0.47 (0.23 - 0.99)*	*0.05*
Singletons	*IVF without ICSI, fresh*	*Term*	*0.83 (0.56 - 1.25)*	*0.38*	*0.84 (0.62 - 1.14)*	*0.27*
Singletons	*IVF without ICSI, frozen*		*0.96 (0.40- 2.31)*	*0.93*	*1.32 (0.73- 2.38)*	*0.36*
Singletons	*IVF without ICSI, frozen*	*>1998##*	*0.92 (0.30- 2.88)*	*0.89*	*0.84 (0.31- 2.24)*	*0.72*
Singletons	*IVF without ICSI, frozen*	*Boys*	*0.80 (0.26- 2.48)*	*0.70*	*1.39 (0.66- 2.92)*	*0.38*
Singletons	*IVF without ICSI, frozen*	*Girls*	*1.48 (0.37- 5.93)*	*0.58*	*1.17 (0.44- 3.11)*	*0.76*
Singletons	*IVF without ICSI, frozen*	*Pre-Term*	*x*	*x*	*2.46 (0.92- 6.58)*	*0.07*
Singletons	*IVF without ICSI, frozen*	*Term*	*1.11 (0.46- 2.67)*	*0.81*	*0.97 (0.46- 2.03)*	*0.93*

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eTable 10 (cont.)

Dataset	IVF Procedure	Sub-Group	Autistic Disorder RR (95% CI)#	Autistic Disorder p-value	Intellectual Disability RR (95% CI)#	Intellectual Disability p-value
Singletons	*ICSI, fresh*		*1.03 (0.65- 1.64)*	*0.90*	*1.23 (0.87- 1.72)*	*0.24*
Singletons	*ICSI, fresh*	*>1998##*	*0.93 (0.54- 1.60)*	*0.79*	*1.35 (0.93- 1.94)*	*0.11*
Singletons	*ICSI, fresh*	*Boys*	*1.04 (0.60- 1.79)*	*0.90*	*1.35 (0.88- 2.05)*	*0.17*
Singletons	*ICSI, fresh*	*Girls*	*1.04 (0.43- 2.50)*	*0.94*	*1.02 (0.58- 1.80)*	*0.95*
Singletons	*ICSI, fresh*	*Pre-Term*	*0.38 (0.05- 2.73)*	*0.34*	*0.50 (0.16- 1.56)*	*0.23*
Singletons	*ICSI, fresh*	*Term*	*1.11 (0.69- 1.79)*	*0.66*	*1.30 (0.92- 1.85)*	*0.14*
Singletons	*ICSI, frozen*		*x*	*x*	*1.84 (0.88- 3.86)*	*0.11*
Singletons	*ICSI, frozen*	*>1998##*	*x*	*x*	*1.88 (0.84- 4.19)*	*0.12*
Singletons	*ICSI, frozen*	*Boys*	*x*	*x*	*1.85 (0.69- 4.93)*	*0.22*
Singletons	*ICSI, frozen*	*Girls*	*x*	*x*	*1.78 (0.57- 5.51)*	*0.32*
Singletons	*ICSI, frozen*	*Pre-Term*	*x*	*x*	*4.00 (1.29-12.43)*	*0.02*
Singletons	*ICSI, frozen*	*Term*	*x*	*x*	*1.22 (0.46- 3.25)*	*0.69*

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eTable 10 (cont.)

Dataset	IVF Procedure	Sub-Group	Autistic Disorder RR (95% CI)#	Autistic Disorder p-value	Intellectual Disability RR (95% CI)#	Intellectual Disability p-value
Singletons	*ICSI, fresh, surgery*		*0.81 (0.11- 5.75)*	*0.83*	*0.53 (0.07- 3.75)*	*0.52*
Singletons	*ICSI, fresh, surgery*	*>1998##*	*0.88 (0.12- 6.27)*	*0.90*	*0.59 (0.08- 4.20)*	*0.60*
Singletons	*ICSI, fresh, surgery*	*Boys*	*1.09 (0.15- 7.78)*	*0.93*	*0.88 (0.12- 6.22)*	*0.89*
Singletons	*ICSI, fresh, surgery*	*Girls*	*x*	*x*	*x*	*x*
Singletons	*ICSI, fresh, surgery*	*Pre-Term*	*x*	*x*	*x*	*x*
Singletons	*ICSI, fresh, surgery*	*Term*	*0.92 (0.13- 6.54)*	*0.93*	*0.62 (0.09- 4.39)*	*0.63*
Singletons	*ICSI, frozen, surgery*		*Not estimable - Too few cases*			

Note: x indicate cells not estimable since too few cases.
Adj model denotes adjusting for birth year, age and sex and additionally adjusting for paternal psychiatric history, maternal psychiatric history and paternal and maternal age;
>1998 Only include children born between 1st January 1999 and 31st December 2007

eTable 11 Comparing the risk for Autistic Disorder and Intellectual Disability, multiples vs singletons.

Exposure	Singletons /Multiples [#]	Cases	Person Years	Adj Rate[##] per 100,000	Model[###]	RR (95% CI)	p-value
Autistic Disorder							
Any IVF	Singletons	54	149,932	24.9	reference group		
	Multiples	49	81,186	46.0	Crude	1.66 (1.13-2.44)	0.010
				46.0	Adj	1.88 (1.28-2.77)	0.001
Spontaneous	Singletons	6,683	33,285,383	13.8	reference group		
	Multiples	173	709,295	15.9	Crude	1.22 (1.05-1.42)	0.010
				15.9	Adj	1.15 (0.99-1.34)	0.068
Relative risk ratio for any IVF vs Spontaneous					Adj	1.63 (1.08-2.47)	0.021

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eTable 11 (cont.)

Exposure	Singletons /Multiples #	Cases	Person Years	Adj Rate## per 100,000	Model###	RR (95% CI)	p-value
Intellectual Disability							
Any IVF	Singletons	101	149,677	60.0	reference group		
	Multiples	79	81,033	91.4	Crude	1.36 (1.01-1.83)	0.041
					Adj	1.49 (1.11-2.00)	0.008
Spontaneous	Singletons	15,178	33,240,234	36.4	reference group		
	Multiples	469	707,727	51.1	Crude	1.46 (1.33-1.60)	<0.001
				51.1	Adj	1.42 (1.29-1.56)	<0.001
Relative risk ratio for any IVF vs Spontaneous					Adj	1.05 (0.77-1.43)	0.747

RR: Relative risk
Multiple include twins and any higher order birth
Rate adjusted for sex, age and birth year,
Crude: Model adjusting for birth year, age and sex only, Adj: Model adjusting for birth year, age and sex, and additionally adjusting for paternal psychiatric history, maternal psychiatric history and paternal and maternal age

eTable 12 **All children**. Distribution of confounders and children characteristics for spontaneuos conceived with and without hormone treatment. Hormone treatment being the only treatment for fertility.

Variable (N: Number of Children)		Spontaneously conceived without hormone treatment	Spontaneously conceived with hormone treatment as the only fertility treatment
Number of Children (% boys)		2,499,096 (51.4)	11,070 (50.7)
Father Psych. History, N (%)		36,255 (1.5)	39 (1.4)
Mother Psych. History, N (%)		46,186 (1.8)	51 (1.8)
Pre-term (before week 37), N (%)		142,402 (5.7)	435 (15.7)
Multiple Birth, N (%)		53,434 (2.14)	606 (21.8)
Birth year, Median (Min-Max)		1994 (1982-07)	2004 (1990-07)
Maternal age distribution, N (%)	<25 25-29 30-34 >34	531,397 (21.3) 885,827 (35.4) 730,243 (29.2) 351,629 (14.1)	744 (6.7) 3,744 (33.8) 4,311 (38.9) 2,271 (20.5)
Paternal age distribution, N (%)	<30 30-39 40-49 ≥50	967,423 (38.7) 1,283,183 (51.3) 226,426 (9.1) 22,064 (0.9)	2,492 (22.5) 7,179 (64.9) 1,297 (11.7) 102 (0.9)
Years of involuntary infertility, Median (10th-90th percentiles)		0 (0-0)	2 (0-7)